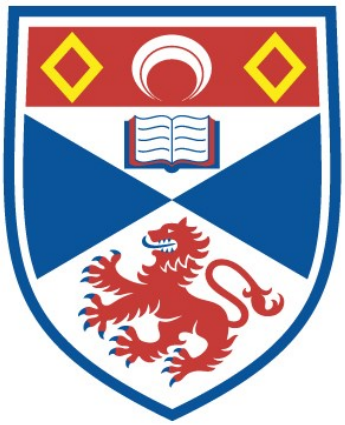
CATALYSIS IN THE HYDROLYSIS OF ESTERS AND RELATED COMPOUNDS

Ian Robertson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1975

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OF

ESTERS AND RELATED COMPOUNDS

A Thesis

presented for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science of the

University of St. Andrews

by

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St. Andrews

September 1975

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SUMMARY

The first chapter is concerned with an investigation into the kinetics and mechanism of the base-catalysed hydrolysis of some substituted phenyl chloroformates. A study of kinetic isotope effects and activation parameters for the hydrolysis of phenyl chloroformate indicates that acetate ion and pyridine act as nucleophilic catalysts. Both the spontaneous and pyridine catalysed hydrolyses of substituted phenyl chloroformates fit a Hammett plot. The effect of solvent polarity and added salt indicates that there is little charge separation in the transition state for hydrolysis. The mechanism of reaction is discussed.

The results of an investigation into the role of nucleophilic and general base catalysis by pyridine and methylpyridines in the hydrolysis of aryl acetates are presented in Chapter 2. Activation parameters for several of these reactions have been determined. The size of the kinetic isotope effect indicates nucleophilic catalysis. Substitution at the 2-position in pyridine prevents nucleophilic catalysis, but a much slower general base catalysed pathway was detected, and it is argued that this occurs even when nucleophilic catalysis appears to be the mechanism.

The third chapter is concerned with the study of micelle effects in the base-catalysed hydrolysis of aryl acetates. It was thought that the base 2(2-hydroxyethyl)-pyridine, in which nucleophilic catalysis due to the nitrogen is effectively blocked by substitution at the two position, would have a tendency to form intramolecular hydrogen bonds within the hydrophobic micelle environment, and hence to enhance the nucleophilicity of the hydroxyl group. No evidence was found to suggest that such a process occurs in the micelle solutions used, but the hydrogen-bonded configuration is shown to be formed

in carbon tetrachloride solution.

Substrate substituent effects in the enzymatic hydrolysis of a series of aryl acetates are investigated in Chapter 4. It is found that substituent effects can change the observed enzymatic mechanism. The possession of a 4-nitro substitutent by the substrate is also shown to be of mechanistic importance. An analogue computer was used to simulate the kinetic results, and factors involved in the change of mechanism are discussed.

DECLARATION

I declare that this thesis is my own composition, that it is based on the results of experiments carried out by me, and that it has not previously been presented for a Higher Degree.

This thesis describes results of research carried out in the Department of Chemistry of the United College of St. Salvator and St. Leonard, University of St. Andrews, under the supervision of Dr. A.R. Butler, between October 1972 and June 1975.

CERTIFICATE

I hereby certify that Ian Robertson has spent twelve terms at research work under my supervision, has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967, No. 1, and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Director of Research

ACKNOWLEDGEMENTS

I would like to thank Dr. A. R. Butler for his help and encouragement in all aspects of this work.

My thanks go to the members of the Chemistry and Physics Departments who have given assistance, and to Mrs. E. J. West for her careful typing of this thesis.

Finally, I gratefully acknowledge a studentship from the Science Research Council.

CONTENTS

CHAPTER ONE

KINETICS AND MECHANISM OF THE BASE-CATALYSED HYDROLYSIS OF SOME SUBSTITUTED PHENYL CHLOROFORMATES

1. INTRODUCTION

Studies of the spontaneous hydrolysis of alkyl chloroformates in aqueous solvents have revealed a change in mechanism from S_N2 to S_N1 along the series methyl, ethyl, propyl and isopropyl[1]. The evidence for the change in mechanism is derived from changes observed in values of the thermodynamic functions $\triangle H^{\ddagger}$, $\triangle S^{\ddagger}$, and $\triangle C_p^{\ddagger}$. A large, positive change in $\triangle S^{\ddagger}$ along the series methyl to isopropyl indicates such a change in mechanism[2]. A plot of $\triangle H^{\ddagger}$ against $\triangle S^{\ddagger}$ results in two distinct straight lines, which do not intersect one another in the region of interest and this evidence also indicates a change in mechanism. However, it has been demonstrated[3] in the hydrolysis of a series of primary alkyl chlorosulphates, that such a relationship cannot always be used unambiguously to establish the plurality, or otherwise, of mechanisms along a series of related compounds. The observed changes in the value of $\triangle C_p^{\ddagger}$, which becomes less negative in the case of a change in mechanism along this series, is, however, thought to be a reliable indication of a change in mechanism[4]. There is also evidence[5] for a similar change in mechanism in the reaction of ethyl chloroformate with a series of substituted anilines.

For the hydrolysis of phenyl chloroformate the evidence indicates an S_N2 mechanism[6], although the rate-determining step may be formation of the tetrahedral intermediate[7], loss of chloride ion, or loss of phenate ion. The observation[8] that the observed rate constant for the reactions of electron-donating substituents, in the hydrolysis of substituted phenyl chloroformates in aqueous

acetone, do not fall on a linear Hammett plot using σ or σ^- constants could, however, be interpreted in terms of a change in mechanism analogous to that which has been shown to occur in the alkyl chloroformate series. The evidence of entropy-enthalpy correlations, of kinetic isotope effects, and of solvent effects for the hydrolysis of the substituted phenyl chloroformates are, however, against such an interpretation. This present study was undertaken in an attempt to clarify the mechanisms by which substituted phenyl chloroformates undergo hydrolysis.

Chloroformates have been extensively used to study reactions involving replacement of chlorine attached to an acyl carbon. Acid chlorides themselves tend to react too quickly for their kinetics to be followed by conventional techniques. The insertion of an oxygen atom, between the hydrocarbon group and the acyl carbon, frequently slows down the reaction rate to within the range which may be conveniently studied. Bimolecular reaction rates for attack at the acyl carbon are dependent on the low electron density on the acyl carbon but, in the presence of an attached ether linkage, the electronegativity of the carbonyl oxygen can be countered by conjugative release from the ether oxygen. In effect, such a process gives initial state stabilization in chloroformates, of a type not possible in the series of related carboxylic acid chlorides.

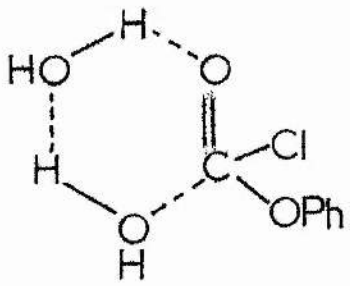
The chemical reactions of chloroformate esters have been extensively reviewed[9,10]. Nucleophilic substitution reactions of these compounds formally parallel those of other types of carboxylic acid esters, although acid-catalysed reactions are not of any major importance. Straightforward hydrolysis of chloroformate esters yields the corresponding alcohol or phenol, hydrogen

chloride, and carbon dioxide, although complications can arise from the further reaction of the liberated alcohol or phenol with the chloroformate to yield a disubstituted carbonate as a side product. The conditions and mechanisms for both the formation and the decomposition of such carbonates are well understood[11,12], and it is possible to ensure that such side reactions do not interfere with the hydrolytic pathways under study. Other recent studies[13,14] have examined the mechanisms of hydrolysis and carbonate formation of substituted chlorothioformate esters.

2. RESULTS AND DISCUSSION

The rates of spontaneous hydrolysis, k(obs), of a series of substituted phenyl chloroformates, with substituents varying from 4-MeO to 4-NO_2, were determined in aqueous dioxan by spectrophotometrically monitoring the absorbance of the substituted phenol released on hydrolysis. A plot of log k(obs) against the Hammett σ constants is linear (Figure 1). The correlation coefficient, r for this plot is 0.991, and thus provides no evidence for a change in mechanism as the substituent is changed.

In furtherance of the mechanistic study of these reactions the rate of hydrolysis of phenyl chloroformate, as a function of the water content in the aqueous dioxan reaction medium, was studied (Table 1). The small effect upon the rate of reaction caused by a change of solvent from pure water to 10% aqueous dioxan indicates a transition state in which there is little charge separation ; it may also indicate formation of a cyclic transition state (1) by the synchronous transfer of a proton from water to the carbonyl group as the chloride ion leaves.



(1)

The linear free energy correlation[15]

$$\log k = \log k_o + mY$$

Figure 1 ; Hammett plot for the hydrolysis of phenyl chloroformates at 25°

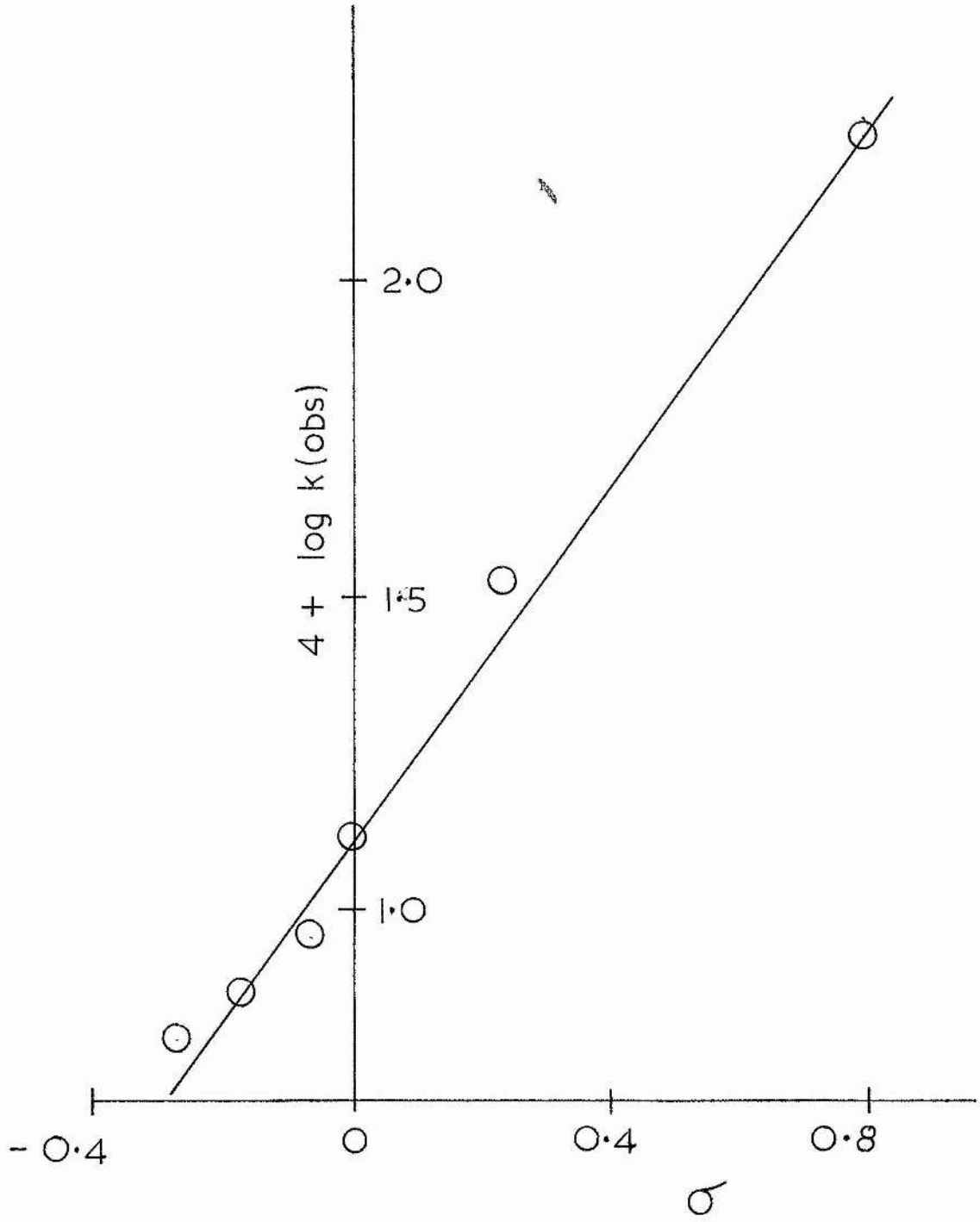


TABLE 1

Variation of k(obs) with water content for the hydrolysis of phenyl chloroformate in aqueous dioxan at 25.0°

% Water (v/v)	10	20	30	40	50	60	70	80	90
10^3 k(obs) s^{-1}	0.34	1.0	1.8	3.1	4.9	6.1	7.8	9.1	10.9

TABLE 2

Hydrolysis of phenyl chloroformate in various acetate buffers at 25°

I = 0.1M $[PhOCO.Cl]_o$ = ca. 10^{-5}M

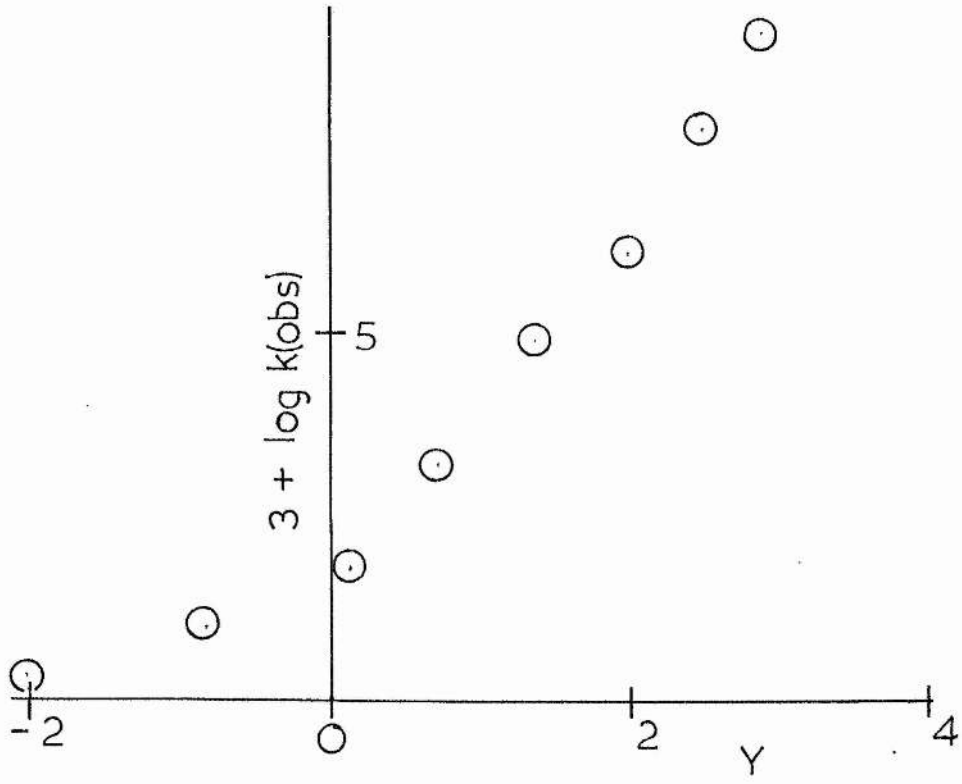
$[OAc^-]$ M	0.05	0.06	0.07	0.08	0.09	0.10
pH	10^2 k(obs) s^{-1}					
4.31	2.10	2.31	2.59	2.84		3.30
4.62	2.28	2.53	2.74	3.04	3.31	3.54
5.03	2.31	2.59	2.72	2.99	3.20	3.45
5.17	2.29	2.56		2.96		3.44
5.31	2.37	2.63	2.81	2.95	3.29	3.43
5.86	2.24			2.86		3.30

where k and k_o are the first order solvolytic rate constants for a certain substance in a solvent and in the standard solvent (80% ethanol) respectively, and m is the parameter measuring sensitivity of the solvolysis rate to Y, the measure of ionising power of the solvent, has been used[16] in an attempt to differentiate between S_N1 and S_N2 solvolytic mechanisms. From the results in Table 1, there is a linear correlation between log k(obs), for less aqueous media, and the values of the solvent parameter Y for aqueous dioxan, although this correlation does not extend into the more aqueous region (Figure 2). The value found for m of 0.193 is substantially different from the values noted[16] for a number of S_N1 reactions. This finding, together with the non-linearity of the plot of log k(obs) against Y over the complete solvent range, is in favour of an S_N2 mechanism. There is also a non-linear relationship between log k(obs) and the values of E_T, a solvent polarity parameter[17], for aqueous dioxan (Figure 3).

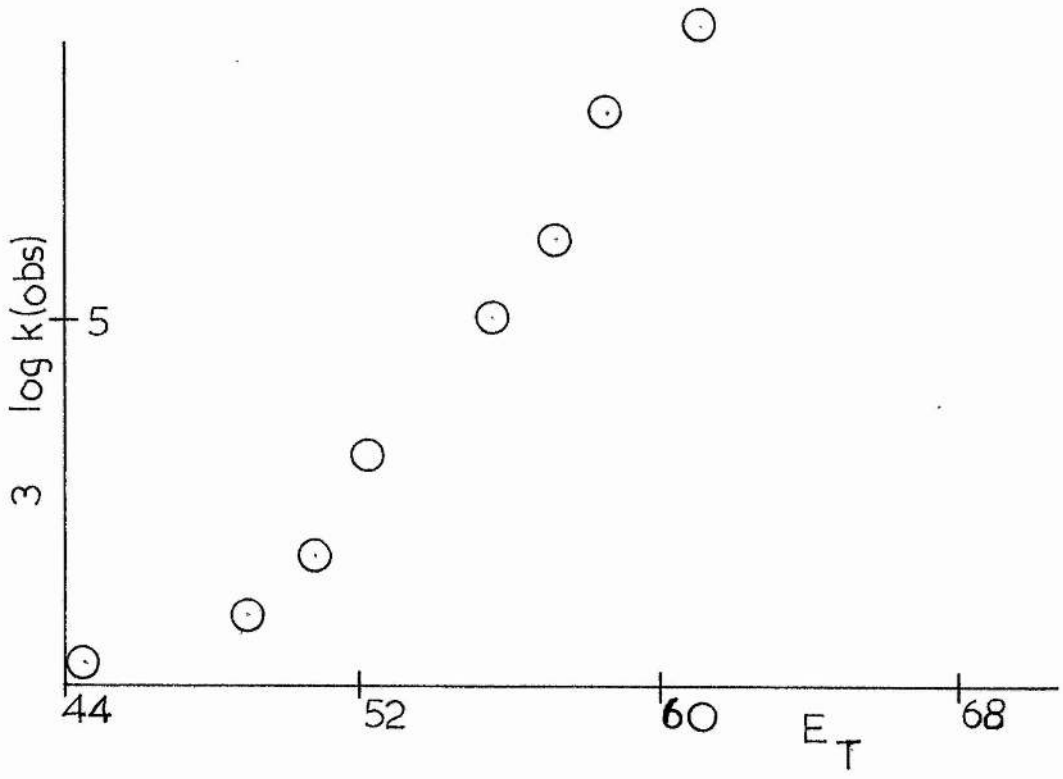
In order to attain a more general understanding of the hydrolytic mechanisms of substituted phenyl chloroformates, the general base catalysis of the reactions were studied. Some thermodynamic studies and an investigation of solvent isotope effects for the reactions were also conducted.

The rates of hydrolysis of phenyl chloroformate in a variety of acetate buffers are presented in Table 2. For reasons of solubility the reaction media contained 10% dioxan ; the ionic strength was maintained at a value of 0.1 M by addition of the requisite amounts of potassium chloride. Interpretation of the results may be achieved by considering the component terms of the observed rate constant ; the spontaneous reaction, k_o, catalysis by hydroxide ion, $k(OH^-)$,

(Figure 2 ; Y plot for the hydrolysis of phenyl chloroformate at 25°)



(Figure 3 ; E_T plot for the hydrolysis of phenyl chloroformate at 25°)



and catalysis by acetate ion, $k(OAc^-)$. It can be seen from Table 2 that, at constant acetate ion concentration, the rate is independent of pH, so that the effect of hydroxide catalysis, $k(OH^-)$, is negligible in this pH region and may be ignored. The average value of $k(OAc^-)$ for the six acetate buffers, given by the slope of a plot of k(obs) against the concentration of acetate ion, is $0.23\ 1\ mol^{-1}\ s^{-1}$. The average value of the intercept, which corresponds to the average value of k_o, is $9.0 \times 10^{-3}\ s^{-1}$. This value does not correspond exactly with the value of $10.9 \times 10^{-3}\ s^{-1}$ obtained in Table 1 due to the difference in ionic strength between the two series.

The rate determinations were repeated for the acetate buffer of pH value 5.17 at two different temperatures, giving values of 5.15×10^{-2} and $5.6 \times 10^{-1}\ 1\ mol^{-1}\ s^{-1}$ for $k(OAc^-)$ respectively (Table 3). These results, and the value of $k(OAc^-)$ at 25.0^o, obtained previously (Table 2), were used in an Arrhenius plot (Appendix 1). The resulting plot is linear and gives a value for $\triangle H^{\ddagger}$ and $\triangle S^{\ddagger}$ of 16.1 k cal mol^{-1} and - 4.9 cal $mol^{-1}\ K^{-1}$ respectively for the acetate catalysed reaction.

The rate determinations were also repeated for an acetate buffer in D_2O, containing 10% dioxan, and the values of k(obs) found are given in Table 4. The pD of the acetate buffer was 5.17 and was determined by use of the relationship[18],

$$pD = pH + 0.4.$$

For reaction in D_2O, $k(OAc^-)$ is $0.20\ 1\ mol^{-1}\ s^{-1}$ and, by comparison with the value of $k(OAc^-)$ in H_2O of $0.23\ 1\ mol^{-1}\ s^{-1}$, obtained from the data in Table 2,

TABLE 3

Hydrolysis of phenyl chloroformate in an acetate buffer (pH 5.17) at different temperatures

$I = 0.1$ M $\quad [PhOCOCl]_o$ = ca 10^{-5} M

buffer contains 10% dioxan

$[OAc^-]$ M	0.04	0.06	0.08	0.10
Temp. °C	10^2 k(obs) s^{-1}			
10.8	0.60	0.70	0.81	0.91
35.0	4.75	5.85	7.00	8.10

TABLE 4

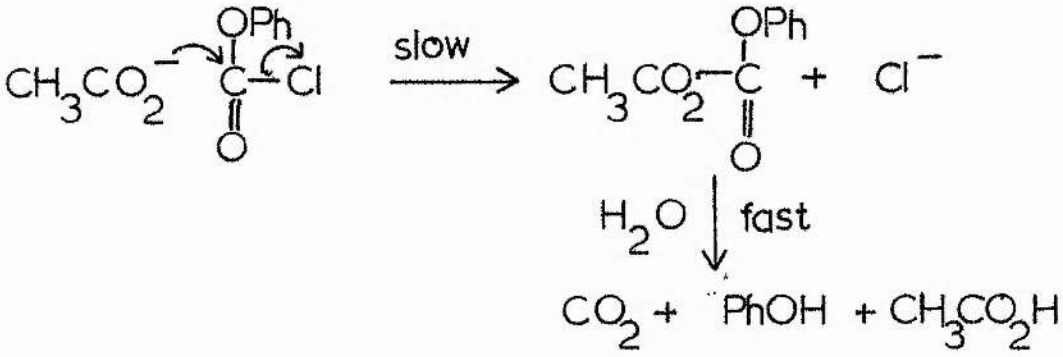
Hydrolysis of phenyl chloroformate in an acetate buffer in D_2O (pD 5.17) at 25.0°

$I = 0.1$ M $\quad [PhOCOCl]_o$ = ca 10^{-5} M

buffer contains 10% dioxan

$[OAc^-]$ M	0.05	0.06	0.08	0.10
10^2 k(obs) s^{-1}	1.47	1.77	2.10	2.50

the solvent isotope effect $k(H_2O)/k(D_2O)$, for acetate catalysis, is 1.1. A solvent isotope effect of this magnitude indicates that the reaction involves nucleophilic rather than general base catalysis. The small negative value for $\triangle S^{\neq}$, obtained from the Arrhenius plot may indicate[19] that no water molecules are directly involved in the formation of the transition state in the reaction. On the basis of these pieces of evidence, the mechanism in Scheme 1 is proposed.



(Scheme 1)

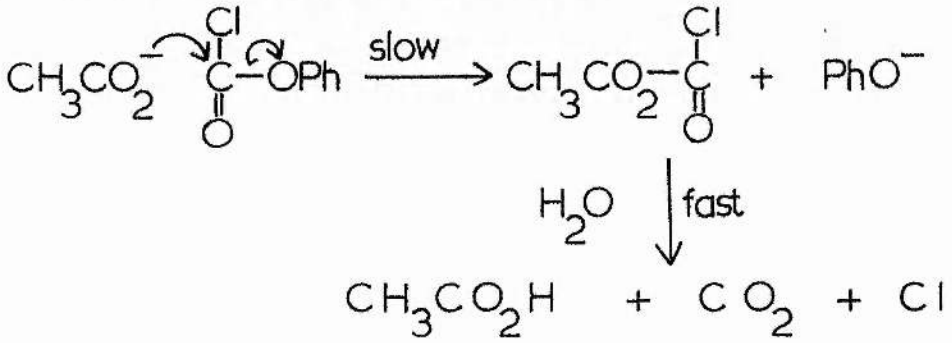
Gold and co-workers[20] have studied the acetate - catalysed hydrolysis of phenyl acetates, where acetate is also acting as a nucleophilic catalyst. The ratio $k(OAc^-)/k_o$ which they observed is similar to the ratio observed in this present study ($21\ M^{-1}$). The value of $\triangle S^{\neq}$ observed for phenyl chloroformate hydrolysis is less negative than that reported for phenyl acetate hydrolysis[21]. It appears, however, that the absolute value of $\triangle S^{\neq}$ is not diagnostic, but large differences should be manifest in the value of $\triangle S^{\neq}$ for the same species acting as a general base and a nucleophile within the same family of reactions.

The work of Gold et al[20, 21] on the acetate-catalysed hydrolysis of a

series of substituted phenyl acetates confirms the dependence of the preferred mechanism on the stability of the leaving phenoxide group ; the greater the stability of the leaving phenoxide group, the greater is the tendency for acetate to act as a nucleophilic catalyst. This trend continues with the more reactive substrate, phenyl chloroformate. It is by no means certain, however, that nucleophilic catalysis in such a reaction occurs to the exclusion of general base catalysis. In such a situation it is possible that one detects only the predominant pathway from the concurrently operating mechanisms for, in the acetate catalysed hydrolysis of an equally reactive substrate, acetic anhydride, the acetate must act as a general base catalyst ; nucleophilic attack would merely regenerate the starting material[22]. This matter is considered in detail in Chapter 2. It has further been observed[23], for acetic anhydride hydrolysis, that the ratio $k(OAc^-) / k_o$ is 1.8. Comparison with the higher ratios obtained for phenyl acetates and phenyl chloroformates may indicate that a different mechanism is operative in the case of acetic anhydride, although such an interpretation must necessarily be cautious, for the spontaneous reactions may have differing mechanisms. In evidence for the need for circumspection, the ratio $k_o(H_2O) / k_o(D_2O)$ for acetic anhydride hydrolysis[23] is 2.9 whilst the same ratio for ethyl chloroformate[24] is only 1.95.

It was assumed, in Scheme 1, that the group displaced by nucleophilic attack is the chloride ion, with the resultant acetyl phenyl carbonate undergoing very rapid hydrolysis. The alternative to this scheme would involve the initial displacement of the phenolate ion, yielding acetyl chloroformate which

subsequently reacts with water in the second step. Such an alternative (Scheme 2) is, however, unlikely for the following reasons. (1) As previously mentioned, substituent effects correlate with σ rather than σ^- and the latter would be required if phenolate were the leaving group. This relationship seems to be a general effect for a number of related reactions[8]. (2) In the alcoholysis of chloroformates[25] and chlorothioformates[13] the products are carbonates and thiocarbonates, and hydrolysis should follow a similar course. (3) Ethyl hydrogen carbonate decomposes very rapidly in aqueous solution with the production of carbon dioxide and ethanol[26].



(Scheme 2)

Another possible mechanism which involves the rate determining step being the reaction of phenyl chloroformate with water, to form a tetrahedral intermediate, is not in accord with the evidence of the solvent isotope effect, which indicates nucleophilic catalysis, or with the interpretation placed upon the value of $\triangle S^{\neq}$. Thus Scheme 1, or some slight variation of it, provides a reaction scheme which is consistent with the available evidence.

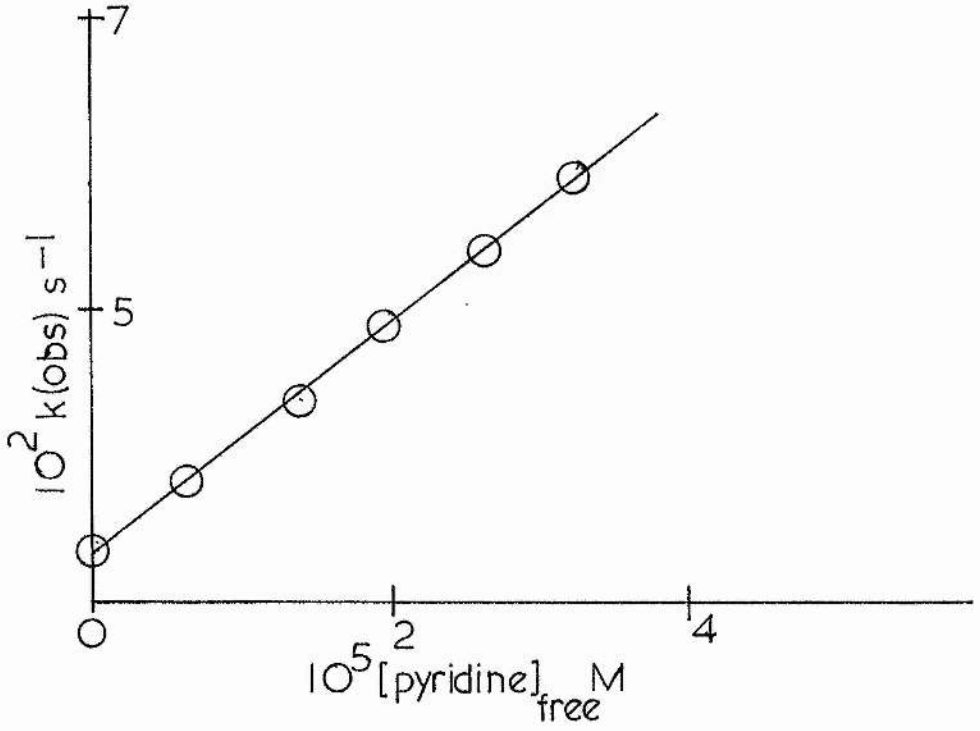
Attempts to study this reaction over an extended pH range, by utilising

borate buffers, were unsuccessful owing to the precipitation of boric esters formed by reaction of phenyl chloroformate with the constituents of the borate buffer, as has been previously noticed[27].

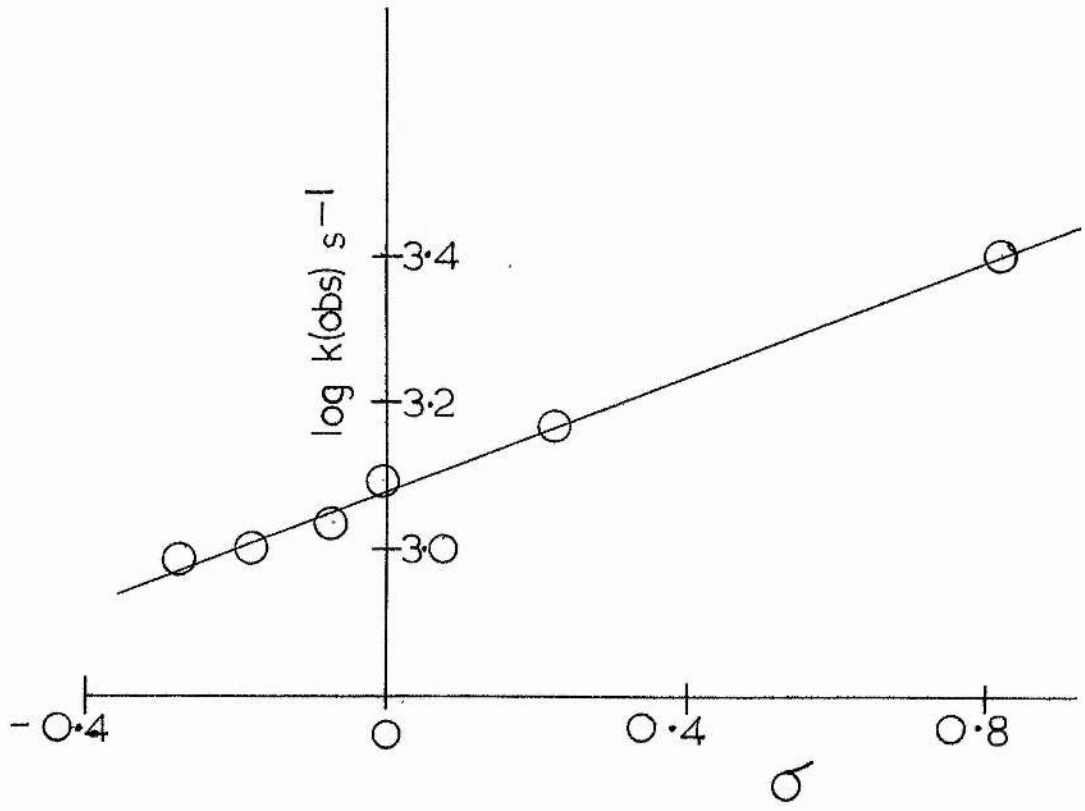
It has been shown[28] that heterocyclic bases catalyse the hydrolysis of acetates. By analogy, such bases should also catalyse the hydrolysis of phenyl chloroformate. The reaction is, however, too fast to follow in a pyridine buffer. Instead, an acetate buffer containing pyridine at low concentration was used. This technique has effectively been used[29] in a study of the pyridine-catalysed hydrolysis of acetic anhydride. The pyridine concentration was changed by dilution of the reaction medium with acetate buffer. The ionic strength was maintained at 0.1 M. It was found that there is a linear relation between k(obs) and the concentration of free pyridine (Figure 4). It was assumed that the pyridine-catalysed hydrolysis of phenyl chloroformate was not susceptible to general acid-catalysis by the pyridinium ion. From the slope of the curve resulting from a plot of k(obs) against the concentration of free pyridine, k for pyridine catalysis is 770 l mol^{-1} s^{-1}. For reaction at 10.0 and 35.0° the values are 390 and 1025 l mol^{-1} s^{-1} respectively. Values of $\triangle H^{\ddagger}$ and $\triangle S^{\ddagger}$, calculated from an Arrhenius plot, were 6.1 k cal mol^{-1} and 24.9 cal mol^{-1} K^{-1}.

The k for pyridine catalysis in D_2O is 480 l mol^{-1} s^{-1} giving a $k(H_2O)$ / $k(D_2O)$ ratio of 1.6. This value of the solvent isotope effect is too high to allow for an unambiguous assignment of the mechanism, although it would be expected that pyridine acts in the role of a nucleophilic catalyst, as

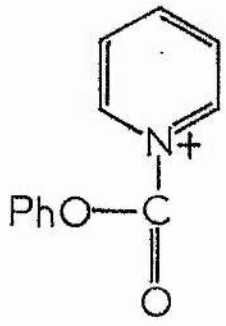
(Figure 4 ; variation of k(obs) with pyridine concentration for the hydrolysis of phenyl chloroformate at 25°)



(Figure 5 ; Hammett plot for the pyridine - catalysed hydrolysis of phenyl chloroformate in 90% aqueous dioxan at 25°)



was found to be the case in a similar study with acetic anhydride[29]. It is generally considered[30], however, that any value in the range one to two indicates nucleophilic catalysis. The large difference in the values of $\triangle S^{\neq}$, between pyridine and acetate catalysis, may indicate a different mechanism of pyridine catalysis, from that of acetate. The displacement of chloride by pyridine would yield the intermediate (2), the hydrolysis of which may be the slow step in the mechanism.



(2)

A scheme of this type would be associated with large solvation changes. There is evidence for such variations from the observed entropy change and kinetic isotope effect.

More evidence, concerning the mechanism of this reaction was obtained by studying the catalysis by pyridine of a series of substituted phenyl chloroformates, using the same experimental method. The results fit a Hammett plot (Figure 5), using σ values, with a slope (the value of which corresponds to ρ) of 0.47. The linear relationship with σ, rather than σ^-,

again indicates that the leaving group in the rate-determining process is not the phenolate ion. The very low value of ρ provides further evidence for this postulate and also indicates that there is very little charge separation in the transition state. The correlation coefficient, r, for the single line shown in Figure 5 is 0.989 and there is thus no evidence for a change in rate-determining step.

The small charge separation in the transition state is also reflected in the small effect[31] of added sodium perchlorate upon the rate of hydrolysis of phenyl chloroformate (Table 5). The effect of adding sodium azide, however, reduces the rate of reaction markedly ; 0.70 M sodium azide reduces the rate of reaction to one tenth of that in the absence of azide. The azide ion, which is a good nucleophile, probably reacts with the phenyl chloroformate to form an azidoformate. Azidoformates have been shown[32] to be fairly unreactive towards hydrolysis and thus the rate of release of phenolate ion is reduced.

(Scheme 3)

An authentic sample of phenyl azidoformate which was prepared was found to hydrolyse very slowly. The experimental evidence available, therefore, is consistent with a mechanism as proposed in Scheme 3.

TABLE 5

Effect of sodium perchlorate on the hydrolysis of phenyl chloroformate in 90% aqueous dioxan at 25.0°

$[PhOCOCl]_o$ = ca. 10^{-5} M

$[NaClO_4]$ M	0	0.03	0.13	0.25	0.50	0.70	1.00
10^3 k(obs) s^{-1}	11.3	11.1	10.8	10.2	8.83	7.76	6.21

3. EXPERIMENTAL

a) Sources, Purification, and Preparation of Materials.

All reagents used in kinetic studies were of AnalaR grade.

AnalaR . pyridine was refluxed with solid potassium hydroxide before being carefully fractionally distilled (b. pt. 115°) through a long Vigreux column.

Reagent grade 1,4-dioxan was refluxed over sodium wire until the surface of the metal was not further discoloured during several hours. It was then carefully distilled (b. pt. 101°) from the sodium, utilising a Vigreux column. Thus purified the dioxan was kept in a frozen state until needed, to prevent peroxide and aldehyde formation[33].

Deuteriated water with a stated deuterium content of 99.7% was supplied by Koch-Light Laboratories.

Preparation of substituted phenyl chloroformates

The substituted phenyl chloroformates utilised in this study are all known compounds[34,35] and were prepared by methods already cited in the literature[36,37,38]. Physical data concerning these compounds is recorded in Table 6, and the general preparative method is given below.

A solution of phosgene (0.14 mole) in toluene (125 ml) was cooled to - 10° in a three-necked flask equiped with thermometer, magnetic stirrer, reflux condenser, and dropping funnel. A solution of the substituted phenol (0.12 mole) in N,N-diethylaniline (0.12 mole) was added, with stirring, whilst maintaining the temperature below 0°. If the substituted phenol was insoluble

in N,N-diethylaniline, anhydrous AnalaR acetone (10-20 ml) was added to achieve solution. The reaction mixture was then kept below 0^{o} for three hours, after which time the temperature was slowly allowed to rise to room temperature. The N,N-diethylaniline hydrochloride formed separated out as a precipitate and was removed by filtration. The reaction mixture, cooled to - 15^{o}, was then stirred vigorously and cold 5% hydrochloric acid (75 ml) was added. The toluene layer was then separated, washed with ice water (75 ml), and dried over anhydrous sodium sulphate. Once dried, the sodium sulphate was removed by filtration, and the toluene was removed from the resulting solution by distillation under reduced pressure. The substituted phenyl chloroformate which remained was then purified by careful fractional distillation under reduced pressure.

Preparation of phenyl azidoformate

Sodium azide (0.65g) dissolved in the minimum quantity of 10% aqueous dioxan was added slowly to a stirred solution of freshly distilled phenyl chloroformate (1.36 g) in dioxan (25 ml). The mixture was stirred for 4 hours and the solvents removed under vacuum. The residue was dissolved in ether, washed twice with cold water, and dried ($MgSO_4$). The ether was removed to leave a liquid, which was assumed to be phenyl azidoformate, ν_{max} 2150 cm^{-1} (N_3). Further purification and identification was not attempted because of the danger of explosion[39].

b) Kinetic Method

One drop of a dioxan solution of the chloroformate (ca. 10^{-3} M) was

added to the buffer contained in a cuvette in the thermostatted cell holder of a Unicam SP 500 spectrophotometer. The change in absorbance with time was recorded, using a Unicam SP 22 chart-recorder and a SP 505 programme recorder. The first-order plots obtained were linear over three half-lives. Rate constants were calculated by the method of Swinbourne (Appendix 2).

TABLE 6

Physical data concerning substituted phenyl Chloroformates

Substituent	B.P.	B.P. Lit.	% Yield	λ (obs)nm	Reference
H -	78-80° (22 mm)	80° (22 mm)	84	274	36
4-Me-	118-20 (40 mm)	108 (30 mm)	89	282	36
3-Me-	95-97 (20 mm)	103 (22 mm)	78	275	37
4-MeO-	132-34 (25 mm)	132 (24 mm)	87	293	34
4-Cl-	114-15 (20 mm)	114 (20 mm)	80	274	38
4-NO_2-	80-81°C(M.P.)	80-1°MP	96	400	36

The wavelengths used were as shown in Table 6. The ionic strength was kept constant by the addition of KCl. Spectra were recorded on a Unicam SP 800 spectrophotometer. A Beckman 'Research' pH meter with calomel electrode and general purpose glass electrode was used for the measurement

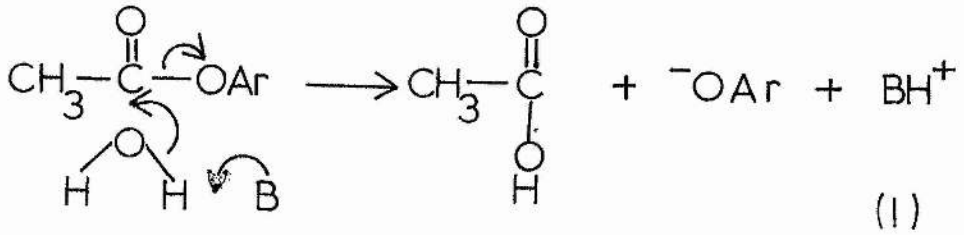
of pH. Values of pD, were obtained by using the relationship, given previously, after Fife et al.[18].

CHAPTER TWO

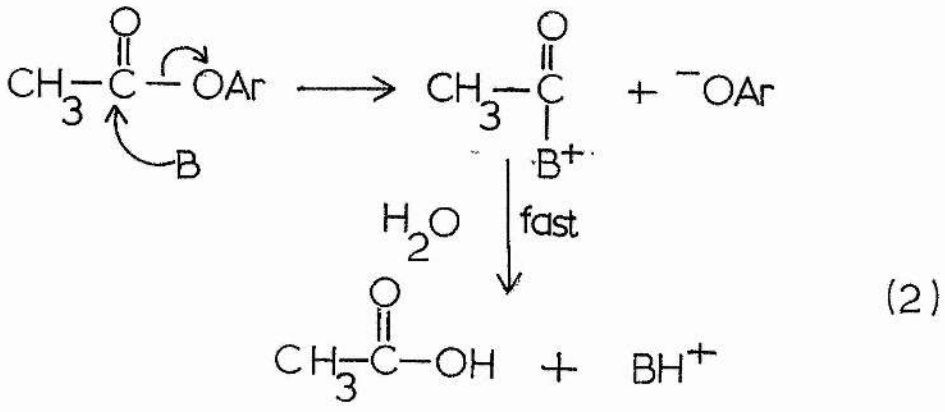
NUCLEOPHILIC AND GENERAL BASE CATALYSIS BY PYRIDINE AND METHYLPYRIDINES IN THE HYDROLYSIS OF ARYL ACETATES.

1. INTRODUCTION

Numerous investigations[40] have been made of base-catalysed ester hydrolysis. In particular, the mechanism of hydrolysis of aryl acetates has been examined in great detail ; these compounds are widely used as substrates in enzymatic studies. The two mechanisms which have been generally proposed for the base-catalysed hydrolysis of such compounds are (1) general base catalysis (Scheme 4), where attack of a water molecule on the carbonyl group of the aryl ester is assisted by partial bond formation to the base (B) and, (2) nucleophilic catalysis (Scheme 5) where the base attacks the ester directly to give an intermediate which is rapidly hydrolysed.



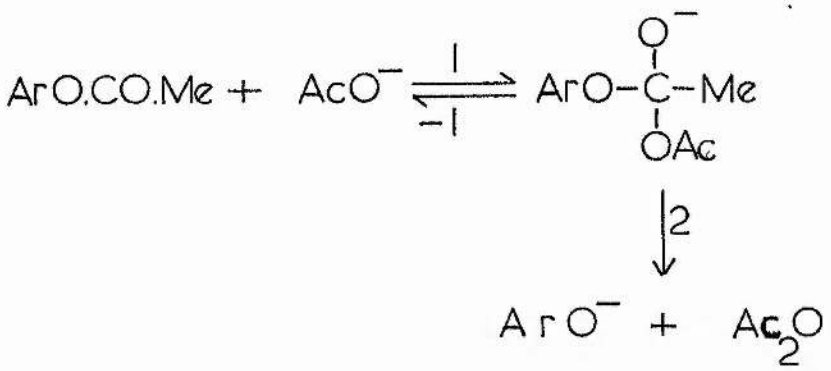
(Scheme 4)



(Scheme 5)

In spite of extensive study, however, it is still not possible to predict with certainty whether any particular catalysing base will act as a nucleophilic or a general base catalyst. The work in this section of the thesis was undertaken in order to investigate the criteria concerned with this plurality of mechanism.

It has previously been shown, by Gold and his co-workers[20,21], that the two mechanisms may occur concurrently in certain instances. The simultaneous existence of the two mechanisms was demonstrated by trapping the intermediate produced in the nucleophilic pathway and comparing the amount of intermediate formed with the amount of reactant consumed. Their general conclusion[20] was that the tendency for nucleophilic catalysis to occur increases as the leaving group becomes better (i.e. the lower the pK_a of the phenol). It was shown, for instance, that the acetate-catalysed hydrolysis of 2,3-dinitrophenyl acetate ($pK_a = 4.96$) is 100% nucleophilic whilst the corresponding reaction for 4-methylphenyl acetate ($pK_a = 10.26$) is 100% general base catalysed. It is for phenols of intermediate pK_a (and hence of leaving tendency) that both pathways may occur ; for example, the acetate-catalysed hydrolysis of 4-nitrophenyl acetate was found to be 70% nucleophilic and 30% general base catalysis. Gold and co-workers[20] suggest that the variation of mechanism can be predicted, to some extent, by consideration of the differences in pK_a values for the acetate ion and the phenoxide ion ; where the phenoxide ion is much more basic than the acetate ion ($\triangle pK_a$ greater than 3), the nucleophilic mechanism becomes insignificant. Gold et al. point out, from consideration of Scheme 6, that the breakdown of the intermediate by the alternative routes -1 and 2 will depend



(Scheme 6)

upon the anionic stability of the alternative leaving groups (acetate or phenoxide) which, for comparisons within a series with close structural similarities in the leaving groups involved, can be equated with the basicity of the species. For the acetate ester of a very acidic phenol, the reverse step (-1) will be relatively insignificant compared with the favoured breakdown by route 2. Under these circumstances, nucleophilic catalysis should be important, although the nucleophilic component will decrease as the quotient $(k_1\ k_2)\ /\ (k_{-1} + k_2)$ decreases.

In terms of such an analysis the correlation of the onset of nucleophilic catalysis with increasing leaving tendency can be understood, although there seems to be no obvious reason why general base catalysis should cease. The analysis of Gold and his co-workers implicitly assumes that general base catalysis occurs with all esters, but that once nucleophilic catalysis becomes possible, it is the predominant pathway. In a review of this topic Johnson[30] quotes Brønsted β values of ca. 0.5 and ca. 0.8 for general base and nucleophilic catalysis. Hence, the nucleophilic pathway is likely to be much more effective than the general base catalysed reaction and it is clear that, except in a few special borderline cases, it would be difficult to detect the general base catalysed reaction when nucleophilic catalysis occurs. Under experimental conditions

one will generally detect only the predominant pathway, as was noted in Chapter 1 ; those reactions involving both pathways must be very fast if the general base catalysis pathway is to be detected. In view of this criterion, this study has necessitated the use of stopped-flow spectrophotometry.

The experimental work designed to facilitate the observation of the two separate pathways was based on the following rationale : pyridine, an effective catalyst in the hydrolysis of acetic anhydride[41], is rendered catalytically inactive by substitution of a methyl group at the 2-position[29,42], due to steric hindrance to nucleophilic attack. The same effect is observed if substitution occurs in the anhydride, for Butler and Gold[43] have shown that pyridine is not a catalyst for the hydrolysis of 2,2-dimethylpropionic anhydride. Pyridine is also a catalyst for the hydrolysis of aryl acetates[44] and if the mechanism is the same then, by analogous reasoning to that above, the ability of pyridine to act as a nucleophile in this reaction can also be removed by substitution at the 2-position. Any general base catalysed reaction which occurs concurrently with the nucleophilic catalysis will then be the only reaction and, under these circumstances, it will be possible to observe and characterise this reaction pathway.

2. RESULTS AND DISCUSSION

The hydrolysis of 4-nitrophenyl acetate in a series of pyridine and 3-methylpyridine buffers, prepared by half neutralisation of the pyridine base with hydrochloric acid, was examined at three temperatures and the results are given in Table 7. From these results it was found that a plot of log k(obs) against concentration of free (i.e. unprotonated) pyridine and 3-methylpyridine is linear in all cases, with no intercept (Figure 6 and 7). The catalytic coefficients, given at the foot of each column in this table, were used to calculate activation parameters for the two reactions (Table 11).

The non-participation of a water molecule in the rate-determining step of a nucleophilic catalytic process means that, as an indicator of nucleophilic catalysis, the ratio $k(H_2O)/k(D_2O)$ should have a value of about unity[40]; this criterion should, however, be used with caution, although it has a fairly wide validity. The rate of pyridine-catalysed hydrolysis of 4-nitrophenyl acetate in D_2O at 25.0^{o} was measured and the results are given in Table 8. The catalytic coefficient is 1.86×10^{-3} l $mol^{-1}s^{-1}$ and $k(H_2O) / k(D_2O)$ for the reaction is thus 1.0. This result provides good evidence that the reaction exhibits nucleophilic catalysis.

Another criterion for nucleophilic catalysis has been proposed by Gold and his co-worker[21] who found that in the acetate-catalysed hydrolysis of aryl acetates, nucleophilic catalysis is associated with a small negative entropy of activation (-10 cal $mol^{-1}K^{-1}$) whilst, for general base catalysed reactions the value is much more negative (-30 cal $mol^{-1}K^{-1}$). The absolute values of $\triangle S^{*}$

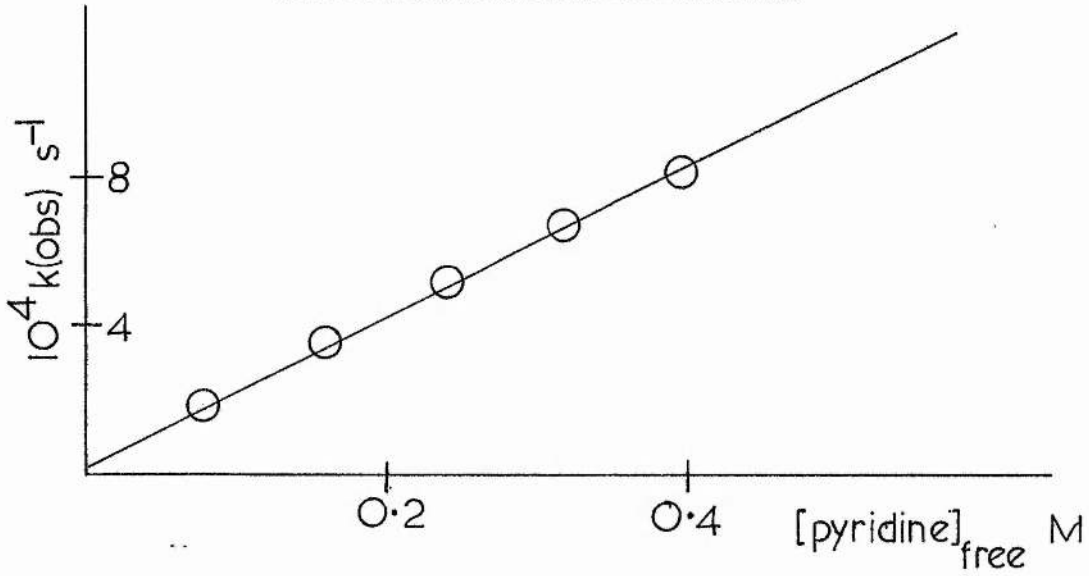
Table 7

The hydrolysis of 4-nitrophenyl acetate in buffered solution at various temperatures

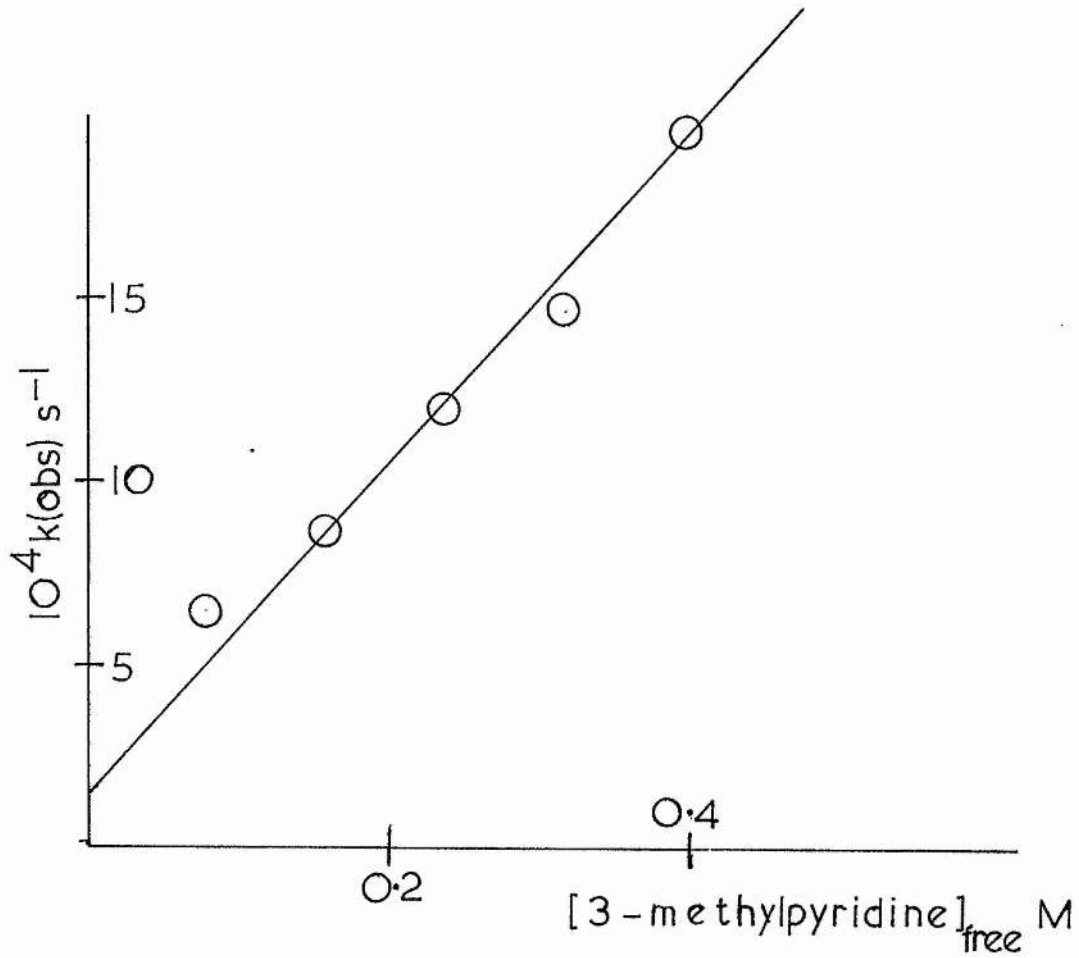
I = 0.5 M $[PNPA]_o$ = ca. 10^{-5} M

	10^4 k(obs) s^{-1}		
T °C	25.5	35.5	45.0
$[C_5H_5N]_{free}$ M			
0.080	1.96	4.56	8.42
0.16	3.68	7.70	
0.24	5.26	10.2	20.3
0.32	6.88	12.8	
0.40	8.32	15.8	30.6
10^3 k l $mol^{-1}s^{-1}$	1.88	3.42	6.80
$[CH_3C_5H_4N]_{free}$ M			
0.080	6.50	8.70	16.0
0.16	8.70	17.7	34.6
0.24	12.0	24.5	46.3
0.32	14.7	30.1	51.4
0.40	19.6	36.5	66.7
10^3 k l $mol^{-1}s^{-1}$	4.46	8.12	14.9

(Figure 6 ; Variation of k(obs) with pyridine concentration for the hydrolysis of PNPA at 25.5°)



(Figure 7 ; Variation of k(obs) with 3-methylpyridine concentration for the hydrolysis of PNPA at 25.5°)



found in this present study do not correspond with those found for the acetate-catalysed reactions. This criterion, although not confirming the mechanism as being nucleophilic catalysis, will, however, be used later to indicate a change in mechanism. In view of the size of the kinetic isotope effect for the reaction, and the previous study[41] of the role of pyridine in the hydrolysis of acetic anhydride, there seems to be little doubt that the present reactions exhibit nucleophilic catalysis.

The substitution of a methyl group at the 2-position of pyridine prevents nucleophilic catalysis for steric reasons, as has been previously mentioned ; if, then, 2-methylpyridine accelerates the hydrolysis of 4-nitrophenyl acetate, the mechanism must be other than nucleophilic catalysis. The pyridine-catalysed reaction of 4-nitrophenyl acetate is not itself a particularly fast reaction, and it was found that with 2-methylpyridine no other reaction, apart from the spontaneous hydrolysis, could be detected. However, if the general-base catalysis pathway is only a small fraction of the total reaction, then that reaction may be too slow to detect. This study, using 4-nitrophenyl acetate, established that the pyridine-catalysed ester hydrolysis is nucleophilic ; similar experimental evidence using a more reactive ester would have required prohibitively large quantities of D_2O to enable the kinetic isotope effect to be measured in a stopped-flow apparatus.

A more reactive ester, 2,4-dinitrophenyl acetate, was chosen for further study. Its rate of hydrolysis in the presence of pyridine, 3-methylpyridine, and 4-methylpyridine was examined by use of a stopped-flow spectrophotometer.

Table 8

Hydrolysis of 4-nitrophenyl acetate in D_2O

at 25°

I = 0.5 M $\quad$ $[PNPA]_o$ = ca 10^{-5} M

$[C_5H_5N]_{free}$ M	0.11	0.26	0.36	0.46
10^4 k(obs) s^{-1}	2.02	4.90	6.80	8.20

Tables 9-11

Hydrolysis of 2,4-dinitrophenyl acetate

catalysed by various pyridine bases

I = 0.5 M $\quad$ $[2,4\text{-DNPA}]_o$ = ca. 10^{-5} M

Table 9

4-methylpyridine

T°C	k (obs) s^{-1}		
$[4\text{-}CH_3C_5H_4N]_{free}$ M	25.2	34.8	44.5
0.036	0.34	0.50	0.78
0.040	0.48	0.71	1.08
0.054	0.51	0.85	1.26
0.072	0.63	0.94	1.43
0.20	1.94	3.22	4.96
0.40	3.38		

Table 10

3-methylpyridine

$T^{o}C$	k (obs) s^{-1}		
	25.2	34.8	44.5
$[3\text{-}CH_3C_5H_4N]_{free}$ M			
0.168	.106	.165	.264
0.336	.210	.323	.505
0.440	.275	.425	.662

Table 11

Pyridine

$T^{o}C$	k (obs) s^{-1}		
	25.2	34.8	44.5
$[C_5H_5N]_{free}$ M			
0.028	0.047	0.088	0.120
0.175	0.281	0.500	0.770
0.250	0.410	0.680	1.070
0.280	0.465	0.775	1.240
0.375	0.595	1.100	1.580

Table 12

Catalytic coefficients for the base-catalysed hydrolysis of 2,4-dinitrophenyl acetate

	k l mol^{-1} s^{-1}		
T°C	25.2	34.8	44.5
Pyridine	1.60	2.70	4.20
3-methylpyridine	6.50	9.90	15.1
4-methylpyridine	8.02	12.9	20.0

Table 13

Activation parameters for the base-catalysed hydrolyses of aryl acetates at 25°

Substrate	Base	$\triangle H^{\neq}$ kcal mol^{-1}	$\triangle S^{\neq}$ cal mol^{-1} K^{-1}
PNPA	Pyridine	11.6	-32 ($\pm$ 1)
PNPA	4-methylpyridine	10.6	-34 ($\pm$ 1)
2,4-DNPA	Pyridine	8.7	-30 ($\pm$ 1)
2,4-DNPA	3-methylpyridine	7.4	-31 ($\pm$ 1)
2,4-DNPA	4-methylpyridine	7.7	-29 ($\pm$ 1)
2,4-DNPA	2-methylpyridine	9.2	-40 ($\pm$ 1)

These results are given in Tables 9-11, the catalytic coefficients for the three bases are collected in Table 12, and the activation parameters for the hydrolysis of both 4-nitrophenyl acetate and 2,4-dinitrophenyl acetate are given in Table 13. From the constant values of $\triangle S^{\neq}$ for the two esters, it is reasonable to deduce that the mechanism of hydrolysis is the same in both cases, implying nucleophilic catalysis in both bases. The change in value of $\triangle H^{\neq}$ between the two esters, becoming less positive in the case of 2,4-dinitrophenyl acetate, is reflected in the increased susceptibility of this ester to hydrolysis.

The sterically hindered base, 2-methylpyridine, was found to act as a catalyst for the hydrolysis of 2,4-dinitrophenyl acetate, albeit at a much reduced rate from that found for other bases. The catalytic coefficients for this reaction at 25.3, 34.9 and 45.3° are 2.80×10^{-3}, 4.93×10^{-3}, and 8.05×10^{-3} l mol^{-1} s^{-1} respectively, and the activation parameters are as shown in Table 13. Gold and his co-workers have observed[21] that a change in mechanism is reflected by changes in the activation parameters. Such changes are seen in the activation parameters given in Table 13 ; the change in both $\triangle H^{\neq}$ and $\triangle S^{\neq}$ clearly indicate a different mechanism and a more negative value for $\triangle S^{\neq}$ corresponds to that observed[21] when the operative form of catalysis changes from nucleophilic to general base. To confirm that general base catalysis occurs with 2-methylpyridine, the solvent isotope effect was measured ; the rate of this reaction is slow enough to permit the use of normal spectroscopic techniques. The molar rate constant for reaction in D_2O is 7.50×10^{-4} l mol^{-1} s^{-1} and so the value of $k(H_2O)/k(D_2O)$ is 3.7. This value is consistent with general base catalysis for, as a water molecule is involved in the rate-determining

step, there is a substantial change in going from H_2O to D_2O. This evidence shows that, although the only detectable pathway of reaction with the closely related, but sterically unhindered, bases is attributable to nucleophilic catalysis, 2-methylpyridine catalyses the reaction via the general base pathway. It seems, therefore, within this series of pyridine bases, that general base and nucleophilic catalysis operate as concurrent pathways, general base catalysis occurring with all the pyridine bases, and that one pathway is not replaced by the other as the leaving tendency of the departing phenoxide changes.

A series of structurally similar amines have been observed[28] to give a linear Brønsted plot for data concerning their catalytic reaction with 2,4-dinitrophenyl acetate. However, for 2,4-dinitrophenyl acetate, a plot of pyridine pK_a for the three pyridines used in this study against log k is not linear, as it is for 4-nitrophenyl acetate catalysis. The pK_a of 2-methylpyridine is very similar to that of 4-methylpyridine[45] (6.02 and 5.97 respectively) and so if 2-methylpyridine could act as a nucleophile the value of k_n would be ca. 8.0 $1\ mol^{-1}\ s^{-1}$ at 25.0^o. The value of k_n/k_{gb} for these bases is therefore ca. 3500. This value well exemplifies the problem of detecting only the predominant, to the exclusion of the subordinate, pathway in the majority of reactions for, as in the case discussed here, when nucleophilic catalysis can occur the general base-catalysed pathway is insignificant.

Part of the work of Gold and his co-workers[20,21], however, argues against the simultaneous involvement of general base and nucleophilic catalysis in the reaction ; namely that a linear relationship was found between log k_{OAc}

and the pK_a of the displaced phenoxide, although the reaction changes from 100% general base to 100% nucleophilic catalysis. If nucleophilic catalysis occurs in addition to the general base-catalysed reaction, then this linearity should be discontinuous. In a preliminary account of this work[20], the analysis is conducted in terms of two intersecting straight lines and it is thought that such an analysis is correct. It has, however, been noted[46] that a mechanistic change does not invariably cause discontinuity in linear free energy relationships, and that the absence of such a change should not be taken as constituting an infallible mechanistic probe.

The hydrolysis of 4-nitrophenyl acetate and 2,4-dinitrophenyl acetate is alkaline solution has also been examined (Table 14), at three differing temperatures. The two reactions have very similar values for $\triangle H^{\ddagger}$ (11.5 and 12.0 kcal mol^{-1} for 4-nitrophenyl acetate and 2,4-dinitrophenyl acetate respectively) at 25.0° and the reactions show a small variation in $\triangle S^{\ddagger}$ (11.5 and 12.0 cal. $mol.^{-1}$ K^{-1} respectively). This difference in entropy is very slight and is probably due, in part, to the role played by desolvation of the nucleophile in the determination of the rate of reaction[47]. The alkaline hydrolysis of substituted phenyl acetates is further studied in Chapter 4, when these esters are used as substrates in enzymatic hydrolysis.

Table 14

Hydrolysis of aryl esters in alkaline solution

I = 0.5 M		$[ester]_o$ = ca. 10^{-5} M		
		k (obs) s^{-1}		
	T°C	25.5	35.5	45.0
	[KOH] M			
PNPA				
	0.050	0.31	0.86	
	0.10	0.78		2.90
	0.20	1.24	3.22	5.59
	0.30	2.16		8.25
	0.40	3.02	6.00	
	0.50	3.98	7.94	
k l mol^{-1} s^{-1}		7.46	14.1	27.0
2,4 DNPA				
	0.050	3.28	6.20	10.6
	0.15	9.15	17.5	32.4
	0.25	15.7	29.5	53.2
k l mol^{-1} s^{-1}		62.0	117	212

EXPERIMENTAL

a) Sources, purification and preparation of materials

All reagents used in kinetic studies were of AnalaR grade.

Deuteriated water, with a stated content of 99.7%, was supplied by Koch-Light Laboratories.

Preparation of nitrophenyl acetates[48,49]

The appropriate nitrophenol (0.1 mol) in carbon tetrachloride (25 ml) was stirred with acetic anhydride (9.5 ml, 0.1 mol) at room temperature for 24 hours, using pyridine (4 ml, 0.05 mol) as a catalyst. After this period, insoluble material, if present, was filtered off, the filtrate was added to ice/water (100 ml) and the mixture was shaken for 5 minutes to hydrolyse the excess of acetic anhydride. Any solid material which separated was filtered off, chloroform (50 ml) was added to the filtrate, and the organic layer was separated. The aqueous layer was again extracted with chloroform (2 x 50 ml), then the combined organic layers were washed with water (100 ml), dried ($CaSO_4$), and evaporated. The nitrophenyl acetates thus obtained were recrystallised twice from appropriate solvents which, together with physical properties of the products, are shown in Table 15.

TABLE 15.

	PNPA	2,4-DNPA
m.p. ^{o}C	39-40	71-72
lit mp ^{o}C	40-41[51]	72[50]
Crystallising Solvent	CCl_4	CCl_4/petrol (bp 60-80^{o})
λ obs nm	400	406

Micro Analysis	C	H	N	C	H	N
Calculated %	53.1	3.9	7.7	42.48	2.65	12.39
Found %	53.2	3.9	7.9	42.21	2.74	12.54

Purification of pyridine bases

The main experimental difficulty in this study was the purification of the pyridine bases, for normal laboratory samples are often very impure. The validity of these experiments requires that, in particular, 2-methylpyridine contains no base unsubstituted at the 2-position as an impurity. Midland-Yorkshire Tar Distillers Ltd. are thanked for samples of 2-, 3-, and 4-methylpyridine which were analysed 99.9 % pure with respect to total base content.

Pyridine, 3-, and 4-methylpyridine were refluxed over potassium hydroxide before being carefully fractionally distilled through a long Vigreux column. The method used to purify 2-methylpyridine was that of Brown et al[50];

2- methylpyridine (13 ml) was refluxed with 14% boron trifluoride in methanol (4 ml), for three hours. Pure 2-methylpyridine was then distilled off, leaving behind the crystalline addition product formed by reaction between the boron trifluoride and the unsubstituted impurities. A second treatment, using the same method, was found to have no effect on the catalytic efficiency of 2-methylpyridine in the hydrolysis of 2,4-dinitrophenyl acetate; it was assumed, therefore, that the sample was sufficiently pure ; had its small catalytic effect been due to other pyridine bases, present as impurities, then there would not

Table 16

Physical properties of pyridine bases

	b.p. ^{o}C	b.p. lit ^{o}C
Pyridine	115	115.6
2-Methylpyridine	129-130	129.4
3-Methylpyridine	142-143.5	144.0
4-Methylpyridine	144.5-146	145.0

have been a change in $\triangle S^{\ddagger}$ and the kinetic isotope effect for this reaction. Physical data for the pyridine bases is given in Table 16.

The buffered solutions were prepared by half neutralisation of the pyridine base with hydrochloric acid. The ionic strength was maintained at a constant value of 0.50 M upon dilution, by addition of potassium chloride.

2. Kinetic method

For the slow reactions, one drop of a solution of the ester in spectroscopic grade acetonitrile was added to the buffer in a cuvette in the thermostatted cell holder of a Unicam SP 500 spectrophotometer. The increase in absorption at 400 (4-nitrophenyl acetate) or 406 nm (2,4-dinitrophenyl acetate) was monitored using a Unicam SP 505 programme controller and an SP 22 chart recorder. First-order rate constants were calculated by the method of Swimbourne (see Appendix 2) and the reaction was first order over at least three half-lives.

For the fast reaction, a Canterbury stopped-flow spectrophotometer was utilised. A solution of ester in water (made 0.001 M in hydrochloric acid, to suppress hydrolysis) was placed in one reservoir, and the buffer in the other. The change in absorption after mixing was displayed on an Advance OS 3000 cathode ray oscilloscope and this trace was photographed using a Polaroid-Land camera. The reaction was found to obey good first-order kinetics.

The values of k(obs) given in all the Tables are the mean of two or three runs. The initial ester concentration in all reactions is ca. 10^{-5} M.

CHAPTER THREE

MICELLE EFFECTS IN THE BASE-CATALYSED HYDROLYSIS OF ARYL ACETATES.

1. INTRODUCTION

The role of hydrogen bonding in the reactions of molecules of biological origin has been extensively studied and reported[51]. In particular, the reaction mechanism of the enzyme α-chymotrypsin appears to involve substantial intramolecular hydrogen bonding between amino-acid residues of serine and histidine[52,53] (see Chapter 4 for a more detailed discussion of α-chymotrypsin studies). The precise functions of hydrogen bonding in biological catalysis are, however, complex and often hard to discern. The work in this section of the thesis was undertaken in an attempt to clarify the mechanisms of such reactions by means of kinetic studies on a model of an enzymic catalytic site.

Monofunctional hydrogen bonds between small molecules have little or no stability in aqueous solution, due to preferential association of the molecules with water. It is postulated[54] that the catalytic site of the enzyme is a hydrophobic region, located within the structure of the enzyme itself ; in such an environment the tendency for intramolecular hydrogen bond formation, between certain base components of the enzyme, would be increased.

In an attempt to simulate a hydrophobic environment analogous to that of an enzyme a model, consisting of a base , 2(2-hydroxyethyl)-pyridine , capable of intramolecular hydrogen bonding and a micelle system containing distinct regions of hydrophobic and hydrophilic character, was utilised. Using this system, the catalytic hydrolysis of a series of nitro-substituted phenyl acetates, frequently used as substrates in enzyme studies[55], was investigated.

As a background to this work, it would seem to be pertinent to outline

the physical and chemical properties of micelles, although it is not proposed to undertake an exhaustive review of micelle work, as such studies have already appeared[56, 57].

Micelles provide a much-studied example of hydrophobic forces in aqueous solution. The structure of micelles is determined by the hydrophobic portions of the constituent detergent molecules which undergo aggregation in order to minimise contact with water. The hydrophilic portions, which may be charged or uncharged, remain at the surface in contact with the aqueous phase. The resulting micelle may be envisaged as roughly spherical and consisting, on average, of between twenty and one hundred aggregated molecules. Anionic detergents normally contain a long hydrocarbon chain terminating in a carboxylate, sulphate, or sulphonate group with potassium, sodium, lithium, or hydrogen as the small, positively charged counterion. Common cationic surfactants are long hydrocarbon chain quaternary amines or pyridines with bromide, chloride or iodide counterions.

In dilute solution (less than 10^{-4} M) micelles exhibit behaviour akin to that of strong electrolytes but, in more concentrated solutions, pronounced deviations from ideal behaviour are observed. The concentration level for departure from ideal behaviour is called the critical micelle concentration (cmc) and it occurs over a narrow concentration range, rather than at a point. Below the cmc, the micellating agent exists mainly as dimers and other small aggregates. The formation of micelles, which is a dynamic, rather than static, equilibrium of the species, breaks up the "flickering cluster" structure of

water[58], resulting in a large positive entropy increase. The primary reason for micellisation is, however, the decrease in the free energy of the system, which results from the preferential self-association of the hydrophobic hydrocarbon chains of the monomeric detergent molecules.

A cross-section through a micelle[59] would reveal a circular hydrocarbon core, from one to three nanometers in diameter, of hydrophobic character, surrounded by a relatively compact region, known as the Stern layer, in which the charged head groups and small counterions are located. Most of the counterions are, however, located in the adjacent Gouy-Chapman electrical double layer. The counterions contained in this layer are completely dissociated from the charged aggregate and are able to exchange with the bulk of the solvent beyond.

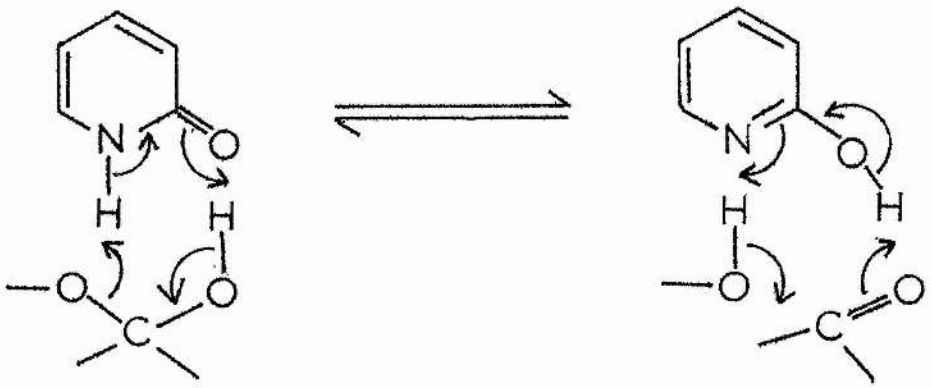
The catalytic effect of micelles upon many types of reactions has been well documented[56, 60]. Such a catalytic effect is explained mainly in terms of two driving forces[61, 62]. Firstly, attractive electrostatic interactions between charges on the micelle and oppositely charged reagents cause an increased concentration of the reagents in the immediate vicinity of the micelle, as compared with the bulk phase. Secondly, catalysis also results from electrostatic stabilization of a charged transition state by the oppositely charged micelle surface. Such catalytic activities are thought to occur in the Stern layer[62]. These assumptions account for the observation that anions, which compete for available binding sites in the Stern layer, are potent inhibitors of surfactant dependant reactions, and also for the fact that no micelle catalysis is observed

in the hydrolysis of neutral reagents and esters[63]. The observation[64] that the salt effect is always larger upon the micelle rate constant than on the uncatalysed reaction can be rationalised by viewing the electrostatic stabilization of the transition state, with respect to the ground state, as one of the primary driving forces for catalysis, and that the large salt effect is a consequence of the weakening of this electrostatic interaction.

It has recently been suggested[65] that another reason for the occurrence of micelle catalysis is the apparent pK_a shift undergone by many nucleophiles, under the action of the surface micelle charge. Such a pK_a shift could, if favourable, result in a large catalytic effect.

2. RESULTS AND DISCUSSION

As part of the investigation of the role of 2(2-hydroxyethyl)-pyridine in ester hydrolysis in the presence of micelles, a brief investigation into the micellular-phase behaviour of two similar pyridine bases was undertaken. The bases chosen for this study were 4-aminopyridine and 2-hydroxypyridine. It has been reported[66] that 2-hydroxypridine exists predominantly as 2-pyridone, whilst the 4-aminopyridine, although potentially tautomeric, exists chiefly in the amino form. There exists, therefore, the possibility of concerted general acid-base catalysis by these bases, as proposed by Swain[67] (Scheme 7). The substituent at the 2-position of a pyridine base ensures, as has already been



(Scheme 7)

shown (Chapter 2), that nucleophilic catalysis due to the nitrogen is sterically hindered, although it is possible that the effect of tautomeric catalysis would overcome the steric hindrance. However, in the typically low dielectric constant environment of a micelle[68] the hydroxy and amino forms should predominate.

The cationic micelle cetyltrimethylammonium bromide (CTAB) was used in the investigation of catalysis in the hydrolytic reaction of 4-nitrophenyl acetate (PNPA) with 4-aminopyridine, and in the reaction of 2,4-dinitrophenyl acetate (2,4-DNPA) with 2-hydroxypyridine (Tables 17 and 18). The reactions were followed by spectrophotometrically monitoring the release of 4-nitrophenol or 2,4-dinitrophenol and were found to exhibit first-order kinetics. A small increase in the rate constant was observed at a concentration of detergent which corresponds to the measured value of the cmc of CTAB (1.0×10^{-3} M, see experimental section). This effect is probably caused by concentration of the reactants within the micelle[61], as compared to the bulk phase, and it is not possible to ascribe such an effect to tautomeric catalysis, or to the operation of any other mechanism. On the basis of the evidence presented such bases in a micelle system have little catalytic efficacy.

The reaction between 2(2-hydroxyethyl)-pyridine and 2,4-DNPA exhibits a linear dependence between the observed first-order rate constant and the concentration of base (Table 19), giving a value of 6.25×10^{-4} l mol^{-1} s^{-1} for k_b. The kinetics of the reaction between 2(2-hydroxyethyl)-pyridine and 2,4-DNPA in the cationic detergent CTAB, and in the anionic detergent sodium lauryl sulphate (Na LS) show little evidence for catalytic action (Table 20 and 21). It is, however, noteworthy that whereas CTAB causes a slight increase in the first-order rate constant in the region of the cmc, due to concentration of reactants in the micelle phase, the anionic detergent NaLS causes a slight decrease. This effect, as has been observed previously[61], is

TABLE 17

Effect of CTAB concentration on the rate of hydrolysis of PNPA in a 4-aminopyridine buffer at 25.1°

pH = 9.15 buffer ratio = ca. 1

pK_a of base = 9.11 $[4\text{-aminopyridine}]_{free}$ = 0.45 M

I = 0.5 M $[PNPA]_o$ = ca. 10^{-5} M.

10^4 [CTAB] M.	0	5	10	15	50	100
10 k(obs) s^{-1}	5.54	5.51	5.61	5.60	5.66	5.65

TABLE 18

Effect of CTAB concentration on the rate of hydrolysis of 2,4-DNPA in a 2-hydroxypyridine buffer at 25.1°

pH = 11.62 buffer ratio = ca. 1

pK_a of base = 11.65 $[2\text{-hydroxypyridine}]_{free}$ = 0.42 M

I = 0.5 M $[2,4\text{-DNPA}]_o$ = ca. 10^{-5} M

10^4 [CTAB] M	0	5	10	15	50	100
10^2 k(obs) s^{-1}	3.85	3.81	3.86	3.92	3.87	3.94

TABLE 19

Effect of buffer concentration on the rate of hydrolysis of 2,4-DNPA in a 2(2-hydroxyethyl)-pyridine buffer at 44.6°

pH = 6.13 buffer ratio = ca. 5

pK_a of base = 5.31 $[base]_{free}$ = 0.710

I = 0.5 M $[2,4\text{-DNPA}]_o$ = ca. 10^{-5} M

$[base]_{free}$ M	0.710	0.568	0.426	0.284
10^4 k (obs) s^{-1}	5.65	4.65	3.90	2.90

$$k_{base} = 6.25 \times 10^{-4} \text{ l mol}^{-1} \text{ s}^{-1}$$

TABLES 20-21

Effect of CTAB (20) and Na LS (21) concentration on the rate of hydrolysis of 2,4-DNPA in a 2(2-hydroxyethyl)-pyridine buffer at 44.6°

$I = 0.5$ M $\qquad [2,4\text{-DNPA}]_o$ = ca. 10^{-5} M

buffer ratio = ca. 5

TABLE 20

$[\text{base}]_{\text{free}} = 0.710$ M

10^4 [CTAB] M	0	1	2	5	10	15	20	50	75	100
10^4 k(obs) s^{-1}	5.65	5.67	5.64	5.68	5.85	6.02	6.01	6.05	6.09	6.13

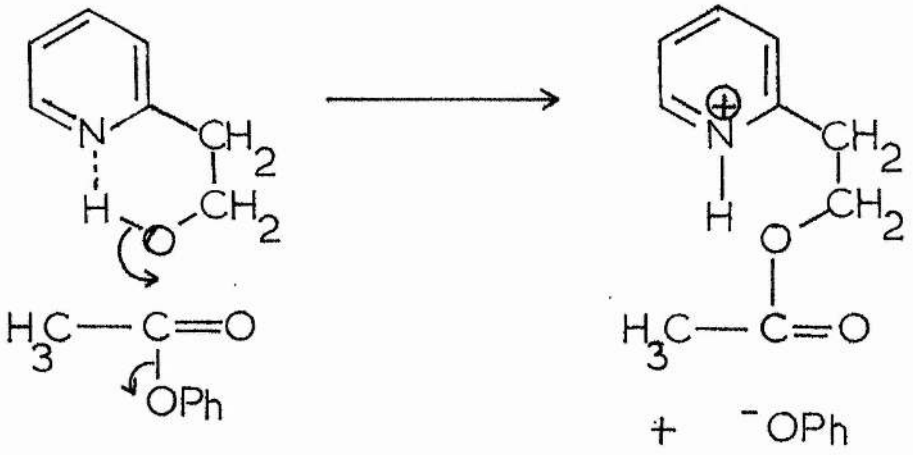
TABLE 21

$[\text{base}]_{\text{free}} = 0.681$ M

10^4 [Na LS] M	0	5	10	15	25	50	100
10^4 k(obs) s^{-1}	5.50	5.53	5.45	5.20	4.80	4.45	4.75

a result of a decrease in the concentration of negatively charged nucleophilic reagents in the anionic micelle phase caused by electrostatic repulsions, and a decreased capacity for solubilisation within that phase.

The absence of catalytic activity in this micellular environment was a disappointment as it was felt that the conformation of the 2(2-hydroxyethyl)-pyridine was such that hydrogen-bonding, in a hydrophobic medium, would lead to the establishment of an unstrained six-membered ring system from the side-chain, and hence to the catalytic mechanism, as depicted in Scheme 8. On the basis of past observations[61, 62], which demonstrated that the rates of



(Scheme 8)

hydrolysis of PNPA and 2,4-DNPA are relatively insensitive to surfactant concentration, it is arguable that only a small increase in the respective rates would be observed in the event of the proposed mechanism (Scheme 8) operating. It was anticipated, however, that any catalytic changes would be of such magnitude as to be easily discernable from the increases caused by the micellular concentration of reactants.

To meet such objections the series of reactions involving 2(2-hydroxyethyl)-pyridine and CTAB were repeated using a different substrate, 4-nitrophenyl hexanoate (PNP He), whose hydrolytic reactions are subject to marked catalysis by micelles[61, 62] (Table 22). The difference in the susceptibility of these two substrates, PNPA and PNPHe, to micelle catalysis is suggested[62] to be due to a large difference in the free energy of transfer from the aqueous to the micelle phase, in favour of PNPHe. It has also been observed that the first order hydrolytic rate constant increases more rapidly than the increase in the CTAB concentration and it is suggested[62] that this phenomena is due to induced micellisation of the CTAB by the PNPHe. However, from the negative evidence in Table 20 it must be concluded that there is no evidence for the premise that a micelle environment induces hydrogen-bonding in the molecule 2(2-hydroxyethyl)-pyridine.

Solution infra-red spectra of a sample of 2(2-hydroxyethyl)-pyridine in a solvent which promotes hydrogen-bonding, carbon tetrachloride, revealed a peak at 3450 cm^{-1}, which was not affected by further dilution of the base by carbon tetrachloride. The position of this peak[69] and its behaviour upon dilution are typical of absorption caused by intramolecular hydrogen bonds. When 2,4-DNPA was added to the solution of 2(2-hydroxyethyl)-pyridine in carbon tetrachloride no spectral change was observed in the ultra-violet spectral region. However, the use of a more active substrate, 2,4,6-trinitrophenyl acetate (2,4,6-TNPA), resulted in the slow appearance of peaks at 345 and 400 nm, which were shown to correspond with release of the parent phenol, with

TABLE 22

Effect of CTAB concentration on the rate of hydrolysis of PNPHe in a 2(2-hydroxyethyl)-pyridine buffer at 44.6°

pH = 6.13 buffer ratio = ca. 5

pK_a of base = 5.31 $[base]_{free}$ = 0.710

I = 0.5 M $[2,4\text{-DNPA}]_o$ = ca. 10^{-5} M

10^4 [CTAB] M	0	1	5	15	25	50	100
10^4 k(obs) s^{-1}	1.93	2.15	2.44	2.75	2.83	2.92	3.13

a first-order rate constant of 6.33×10^{-3} s^{-1}. Monitoring the absorbance in the infra-red region, due to intramolecular hydrogen-bonding, revealed a corresponding increase in the transmittance of this peak during the course of the reaction, although experimental difficulties prevented any kinetic data being obtained. No spectral changes occured when 2,4,6-TNPA was added to carbon tetrachloride as a blank reaction. Similarly, two structural analogues of 2(2-hydroxyethyl)-pyridine, namely 2,6-lutidine and 2-phenylethanol, produced no spectral changes in two similar separate experiments. The two analogous compounds were then used, in equimolar concentrations, in the same kinetic run, because absorption due to trinitrophenol might have resulted from nucleophilic attack of the hydroxy compound, followed by proton transfer to the nitrogen of a different molecule to give an ion pair. However, no change in the spectrum was observed. Product identification using N.M.R. showed that reaction between 2,4,6-TNPA and 2(2-hydroxyethyl)-pyridine in carbon tetrachloride gave O-acetyl-2(2-hydroxyethyl)-pyridine.

In considering the possible mechanism of the reaction between 2,4,6-TNPA and 2(2-hydroxyethyl)-pyridine in carbon tetrachloride it should be noted that a substituent in the 2-position of the pyridine diminishes the ability of the nitrogen to participate in nucleophilic catalysis, as witnessed by the absence of spectral change in the reaction between 2,4,6-TNPA and 2,6-lutidine in carbon tetrachloride (see also Chapter 2). It would seem, therefore, that the tendency for the formation of hydrogen bonds increases on transference of the reaction from aqueous micelle systems to non-polar solvents. The experimental

evidence, in particular the product analysis, can best be accounted for in terms of the mechanism in Scheme 8.

In view of this conclusion it must be questioned whether the 2(2-hydroxyethyl)-pyridine was adsorbed, under the reaction conditions used, into the hydrophobic regions of the micelle system sufficiently for the promotion of hydrogen bond formation in the base. Such a problem, concerning the location of the base in the micelle structure, could probably be resolved by the use of N.M.R. techniques[70].

3. EXPERIMENTAL

a) Sources, purification, and preparation of materials

All materials used in kinetic studies were AnalaR grade.

The micelles used in the present study, CTAB and NaLS, were purified by the method of Duynstee and Grunwald[50].

Sodium lauryl sulphate (25 g) from Koch-Light Laboratories was dissolved in 95% ethanol (350 ml) and heated. After filtration and cooling, white blades were obtained. This procedure was repeated twice : the final product was dried in a vacuum desiccator.

Cetyltrimethylammonium bromide (Kock-Light Laboratories) was shaken with anhydrous ether, filtered, and dissolved in the minimum amount of hot methanol. Cooling produced a crystalline mass which was then filtered. The resulting crystals were then redissolved in methanol, anhydrous ether was added, and the solution gently warmed to dissolve the CTAB. The white crystalline product obtained upon cooling this solution had a melting point of 227-235^{o} (lit[50], 227-235^{o}).

Purification of the pyridine bases was achieved by the following methods.

(i) Laboratory grade 2(2-hydroxyethyl)-pyridine (Koch-Light Laboratories) was purified by careful fractional distillation (b.p. 118^{o}, 15 mm Hg).

(ii) A Koch-Light Laboratories pure sample of 2-hydroxypyridine was stirred and heated with sodium hydroxide for two hours, and then carefully

fractionally redistilled (181-184°, 24 mm Hg). The resulting solid had a melting point of 104-105° (lit. = 105-107°)[66].

(iii) A Koch-Light Laboratories pure sample of 4-aminopyridine was further purified by recrystallisation from ethanol ; m.p. 162-163° (lit.[66] 161-162°).

The preparation of 4-nitrophenyl acetate, 2,4-dinitrophenyl acetate, and 2,4,6-trinitrophenyl acetate was achieved by the method cited in the experimental section of Chapter 2.

The method of Romsted and Cordes[62] was utilised in the preparation of 4-nitrophenyl hexanoate. Hexanoic acid (6.4 g) in benzene (50 ml) was added dropwise to a refluxing solution prepared by mixing 4-nitrophenol (6.95 g) in tetrahydrofuran (75 ml) and dicyclohexylcarbodiimide (12.5 g) in benzene (50 ml). The reaction mixture was refluxed for 24 hours, filtered, then washed six times with 5% sodium bicarbonate solution, once with 0.1 M hydrochloric acid, and once with water followed by drying over calcium sulphate. The solvent was then removed and the reaction product was placed on a column containing silica gel (400 g), and eluted with hexane containing increasing quantities of ether. The product was eluted when the ether content reached 10% by volume. The residue, obtained by removal of the solvent, was carefully fractionally distilled under reduced pressure to yield 4-nitrophenyl hexanoate (b.p. 146-148°, 2 mm Hg ; yield = 38% (5.45 g)).

Determination of surfactant critical micelle concentration (cmc.) values

A method[67] has been developed for the determination of surfactant cmc. values using the spectroscopic shifts observed in the spectrum of N,N -diethylaniline. Upon the formation of micelles, the organic compound is readily absorbed into the hydrophobic interior. As a result, the spectrum of the compound shows a distinct shift in its absorption maxima at the cmc ; the shift in N,N -diethylaniline corresponds to a change from aqueous solution to an environment which is very similar to that of n-octane (values for N,N-diethylaniline are :- λ_{max} for H_2O = 253 nm, λ_{max} for n-octane = 261 nm). The solutions used in this determination were those prepared for the kinetic work and were thermostated at the appropriate temperatures before use. The experimental conditions were thus analogous to those in the kinetic runs. The N,N -diethylaniline was of spectral grade and was freshly distilled before use, because prolonged exposure to air results in oxidation.

N,N -diethylaniline (0.002 ml in 6 ml of surfactant solution) was added to the experimental solutions which were then thermostatted at the appropriate temperature using a Unicam SP 875 thermostatted heating block in the cell compartment of a Unicam SP 800 spectrophotometer. The spectrum of the solution was then run, and the position of the maximum absorption was noted. The λ_{max} value increases sharply at the cmc and the following cmc values, which are in good agreement with those determined by other methods[62], were determined ; cmc for NaLS = 8.0×10^{-4} M ; cmc for CTAB = 1.0×10^{-3} M.

All values at 44^{o}.

The rate constants observed for micelle catalysis are quite strongly affected by changes in the background ionic strength, especially for cationic micelles, where such changes might involve a change in the concentration of counterions (e.g. the kinetics of a CTAB catalysed hydrolysis are affected by the background concentration of bromide ion). To overcome such effects, the total concentration of bromide ion in the experimental solutions involving CTAB was maintained at 0.03 M by addition of sodium bromide, and the total concentration of sulphate in NaLS solutions was maintained at 0.03 M by use of sodium sulphate. The ionic strength of the solutions was maintained at an overall value of 0.5 M by addition of sodium chloride. This salt is used instead of potassium chloride because the latter causes precipitation of NaLS. The pyridine base present in the experimental solutions acted as a buffer, and pH values were determined by use of a Beckman Research pH meter equipped with a general purpose and a calomel reference electrode.

N.M.R. identification of the product of the reaction between 2(2-hydroxyethyl)-pyridine and 2,4,6-TNPA

Equimolar quantities (0.02 mol) of 2,4,6-TNPA and 2(2-hydroxyethyl)-pyridine were refluxed in carbon tetrachloride (25 ml) for 24 hours under an atmosphere of nitrogen. The solution was then filtered to remove 2,4,6-trinitrophenol, and was then washed once with cold dilute sodium bicarbonate solution and three times with ice/water (3 x 50 ml) ; the solution was then dried over anhydrous calcium sulphate. The carbon tetrachloride was then removed

under reduced pressure, and the resulting viscous liquid was identified as O-acetyl-2(2-hydroxyethyl)-pyridine by N. M. R. studies. The machine used in these studies was a Varian HA-100 spectrometer. The spectrum of the O-acetyl-2(2-hydroxyethyl)-pyridine in carbon tetrachloride clearly shows, by comparison with the spectrum of the parent pyridine, the disappearance of the peak due to the proton of -O-H(at 5.25δ), and the appearance of a CH_3- peak (at 2.04δ).

(b) Kinetic method

For the slow reactions, one drop of a solution of the ester in acetonitrile was added to a cuvette, containing the experimental solution, in the thermostatted cell holder of a Unicam SP 500 spectrophotometer. The increase in absorption at 400 (PNPA and PNPHe) or 406 nm (2,4-ONPA) was monitored. First-order rate constants were calculated by the method of Swinbourne (Appendix 2) and the reactions were first-order over at least three half-lives.

The reactions of 2-hydroxypyridine and 4-aminopyridine were monitored using a Canterbury stopped-flow spectrophotometer, and the procedure is identical to that given in the experimental section of Chapter 2.

CHAPTER FOUR

THE α -CHYMOTRYPSIN CATALYSED HYDROLYSIS OF SOME SUBSTITUTED ARYL ACETATES

1. INTRODUCTION

The proteolytic enzyme, α-chymotrypsin (α-ct) occurs in the pancreatic juice of mammals as an inactive precursor . chymotrypsin A, which is converted into the active enzyme in the duodenum. The active enzyme, whose biological function is almost certainly protein degradation in digestion, has a specific three-dimensional structure which is essential for catalytic activity and which consists of three polypeptide chains linked by two disulphide bridges. The molecular weight of α-ct is 24, 800, and the enzyme contains 241 amino acid residues.

Many kinetic and mechanistic studies have been performed on α-ct, the aim being to arrive at an understanding of the detailed molecular mechanism. In order to achieve this objective the amino acid residues which are essential for catalysis, and the role which each of these groups play in the catalytic process, must be understood. However, despite the prolific work of chemists and biochemists, which has led to the identification of the amino acid residues involved in catalysis, the mechanism of the proteolytic process has, as of yet, not been unambiguously defined. Several mechanisms, which are reviewed briefly below, have been proposed and it is noteworthy that all of these schemes appear to be supported by good experimental evidence. In view of the disparities between the proposed mechanisms it was felt that it might prove fruitful to examine the effect of changes in the steric and electronic structure of the substrate upon the rate of the enzyme catalysed reaction. In more detail, this section reports the results of a study of the α-ct catalysed hydrolysis of a wide

range of substituted phenyl acetates. Because of the different reactivities encountered across this series, these studies have involved the use both of conventional and of stopped flow techniques.

The three-dimensional structure of the enzyme has been determined, by x-ray crystallography, by Blow and co-workers[53], and the sequences of amino acids and their spatial relationships are thus well known. Studies[71] of the pH effect upon the catalytic coefficients with a wide range of substrates have revealed the involvement of a histidine residue. This behaviour is shown by both amide and ester substrates. Photooxidation, inhibition techniques, and X-ray data have indicated[72] that the residue involved is histidine-57. The same studies have also shown the involvement of serine-195, and model compounds incorporating serine and histidine have shown signs of characteristic enzymatic activity[73]. The X-ray studies[53] have also demonstrated the existence of hydrogen bonding between aspartic acid-102 and the N-terminal residue of isoleucine-16, and it is suggested that this ion pair relation maintains the conformation of the active site. It is important to realise that residues widely separated in the amino-acid sequence may be quite closely located within the three-dimensional spatial structure of the enzyme (as in the foregoing example). The majority of groups which are not directly involved in catalytic action perform the role of shaping the hydrophobic cavity within which catalysis takes place. The nature of this active site also gives the enzyme its characteristic substrate specificity.

Whitaker and Jandorf[74] treated α-ct with dinitrofluorobenzene at pH 10.7 (conditions under which only the imidazole groups of histidine residues react with the reagent) and showed that reaction of one histidine residue

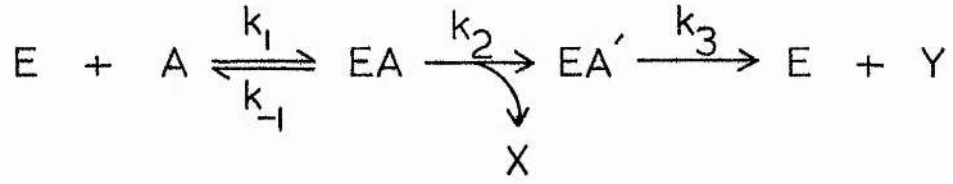
per molecule could cause complete loss of catalytic activity. Similarly, Schramm[75] showed that the inactivation of chymotrypsin by 1,4-dibromo-2-phenylacetoin was caused by the reaction of this reagent with histidine. The evidence is, therefore, that each molecule of enzyme contains only one specific catalytic site.

Similarly, inhibition of the enzyme is achieved[76] by reaction with dialkylfluorophosphates, which selectively react with the hydroxymethyl group of a serine residue. Analysis of the product showed one dialkylphosphoryl residue per molecule in the inactive product. Furthermore, if 4-nitrophenyl acetate (PNPA) reacts with α-ct under acid conditions, an intermediate may be isolated in which the acetyl group is covalently bound to the enzyme. Other acyl-chymotrypsins, prepared in a similar way, may be isolated and in each case there appears to be an ester link between serine-195 and the acyl-residue[77].

The residues involved in catalysis, and the physical structure of α-ct are thus well known and understood : the roles of these residues are, unfortunately, less well understood. As is the case with other enzymes, the active region of α-ct constitutes a peculiar environment in which the reactions of the substrates may take place. From specificity considerations alone it may be supposed that a hydrophobic or non-polar region is present - a region which is comparable to that which is found in the interior of micelles. Indeed, workers have compared micelles containing hydroxy-groups[78] (as a model of serine proteinases) and micelles containing imidazole[79] (as a model of a histidine residue) to the catalytic action of enzymes. Some studies[78] have duplicated the three-stage kinetics, including the rapid burst stage, typical of enzyme

reactions. The catalysis of phenyl ester cleavage by cycloamyloses has also been shown[80] to provide a remarkable model for enzymatic specificity. This specificity enables the enzyme to form a "hydrophobic link" with the aromatic ring of the specific substrates. The most important consequence of the peculiar nature of the active site environment is, however, that the reactivity of certain groups is greatly enhanced. The hydrolysis of acetyl-chymotrypsin to yield acetic acid and free enzyme proceeds, for example, much more readily than would be expected for an ordinary O-acetyl derivative. The reactions of the acetyl-enzyme proceed no faster than would be expected, however, in a medium in which the protein is denatured and the active site destroyed[81] (e.g. 8 M urea).

The minimal mechanism for the enzyme must, however, involve the steps shown in Scheme 9. The first stage consists of the formation of the



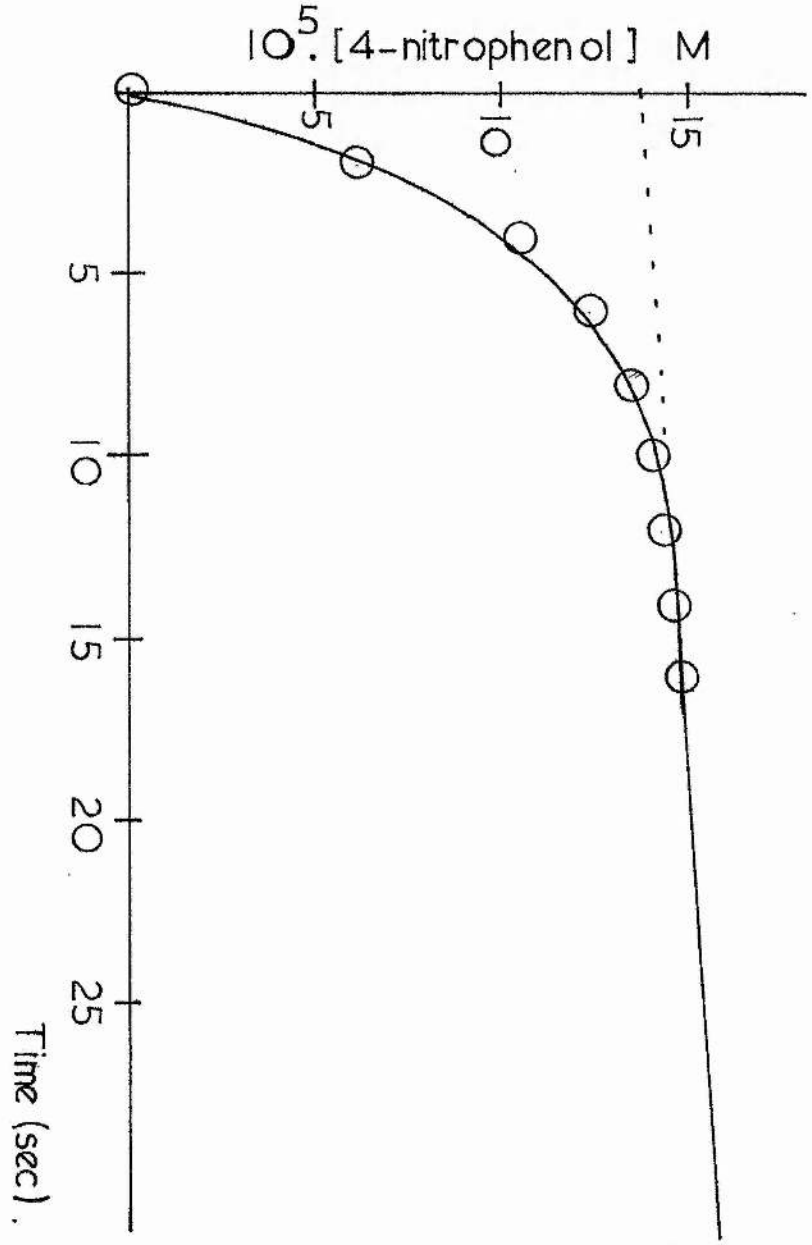
(Scheme 9)

Michaelis-Menton complex (EA). The next stage involves the acylation of serine-195, and the first product X (which is a phenol if the substrate used is phenyl acetate) is released. The final process is the deacylation reaction consisting of reaction of the acyl-enzyme with some nucleophilic species, usually water, giving the second product (acetic acid in the case of phenyl acetate). Such a scheme is used, with simplifying assumptions, as the basis

for the Michaelis-Menton treatment of a single-substrate enzyme-catalysed reaction[82]. This kinetic approach, which has been of great value in the elucidation of general enzyme mechanisms, has been extended in scope by many workers[83]. The kinetic results obtained in this study were analysed using the approach of Gutfreund (Appendix 3), whose treatment allows the separation of the various kinetic parameters.

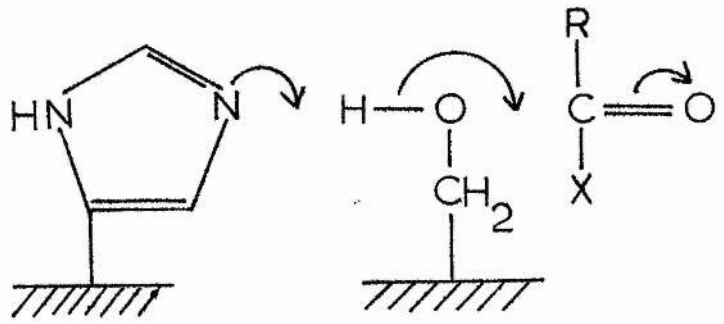
Evidence in favour of the proposed minimal mechanism (Scheme 9) has been observed by Bender and his co-workers[84]. Hartley and co-workers[85] observed that the liberation of 4-nitrophenol during the α-ct catalysed hydrolysis of PNPA proceeds with an initial fast burst, which is then followed by a steady-state release of the phenol (see Figure 8). The observation of this burst necessitates the use of stopped-flow spectrophotometry (although certain substrates have pre-steady-state periods which last several minutes[86]). The kinetic pattern of this reaction is interpreted as being due to the rapid formation of the acyl-enzyme intermediate (EA'), which corresponds to complete acylation of the enzyme with consequent liberation of 4-nitrophenol : further formation of the phenol is then controlled by the relatively slow hydrolysis of the acyl-enzyme to yield free enzyme. Spectral[87] and pH[88] studies have shown that the acyl-enzyme intermediate is a distinct intermediate, as opposed to a Michaelis-Menton complex. Steady-state studies[54, 89] have shown that most ester substrates which react with α-ct do so with the same steady-state rate-constant - an observation which indicates that these substrates react via a common intermediate.

(Figure 8 ; liberation of 4-nitrophenol during the α-ct catalysed hydrolysis of PNPA at 25.3°)



Various approaches, such as studies of substrate binding[90], titration experiments, and spectral changes [91] have shown that observed catalytic parameters are dependent upon an acidic group of pK_a 8.5 and a basic group of pK_a 6.8 (although care and special procedures are needed when correlating the observed pK_a of the enzymic residues with known pK_a values ; as with similar hydrophobic regions within micelles, large shifts are often observed in pK_a values[78]). The acidic group is thought to correspond to isoleucine-16, which is probably involved in hydrogen bond formation with the carboxylate group of aspartic acid-194. The loss of activity at high pH is thought to be due to denaturation of these bonds, although it has also been suggested[92] that the loss of α-ct activity is governed by another α-amino group, alanine-149. Either of these interactions would, however, hold the enzyme in its active conformation ; denaturation results when the amino-group of isoleucine-16 is deprotonated, and the conformation changes to one in which the substrate can no longer be bound.

The group of pK_a 6.8 has, until recently, been identified as that of the imidazole group of histidine-57, which was thought to activate the serine hydroxyl group through general-base catalysis (Scheme 10). There is, however, some question as to whether histidine-57 acts as a general-base or as a nucleophilic catalyst in this reaction. The experimental evidence is as follows :- 1) The values observed[93] for the ratio $k(H_2O)/k(D_2O)$ are in the region of two to three, which signify that proton transfer is involved in the rate determining step. 2) Substituent effects in various substrates indicate[30] the existence of a



(Scheme 10)

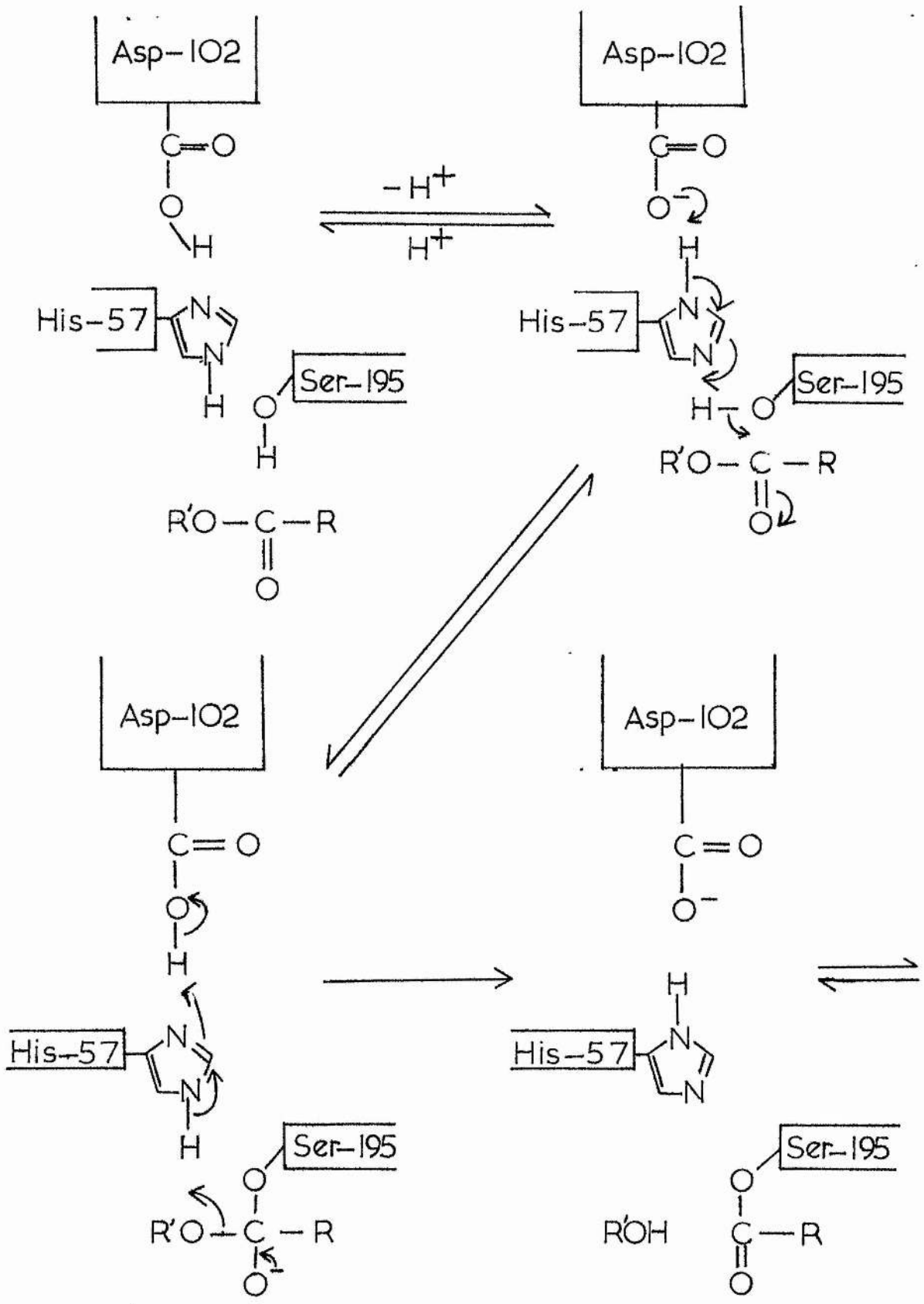
tetrahedral intermediate, and evidence shows that the formation of this intermediate is rate-determining[94]. 3) Substituent effects in aromatic substrates give good correlation with positive Hammett constants, indicating the involvement of an electron-deficient centre during catalysis[55].

The evidence above seems to strongly indicate the operation of general base catalysis but it has been observed[95] that an amide substrate well suited to anchimeric assistance was hydrolysed many orders of magnitude slower by α-ct than were other amides. It must therefore be concluded that the general base explanation is not completely satisfactory. Most mechanisms, however, involve general base catalysis and have as a rate determining step proton transfer. The mechanisms can be divided into those in which a hydrogen-bonding between histidine-57 and serine-195 is postulated (e.g. Wang[96], Blow[53,97]), and those in which no such bond is postulated (e.g. Bender[98]). Composite mechanisms, which combine the best features of previous proposals, have also been advanced

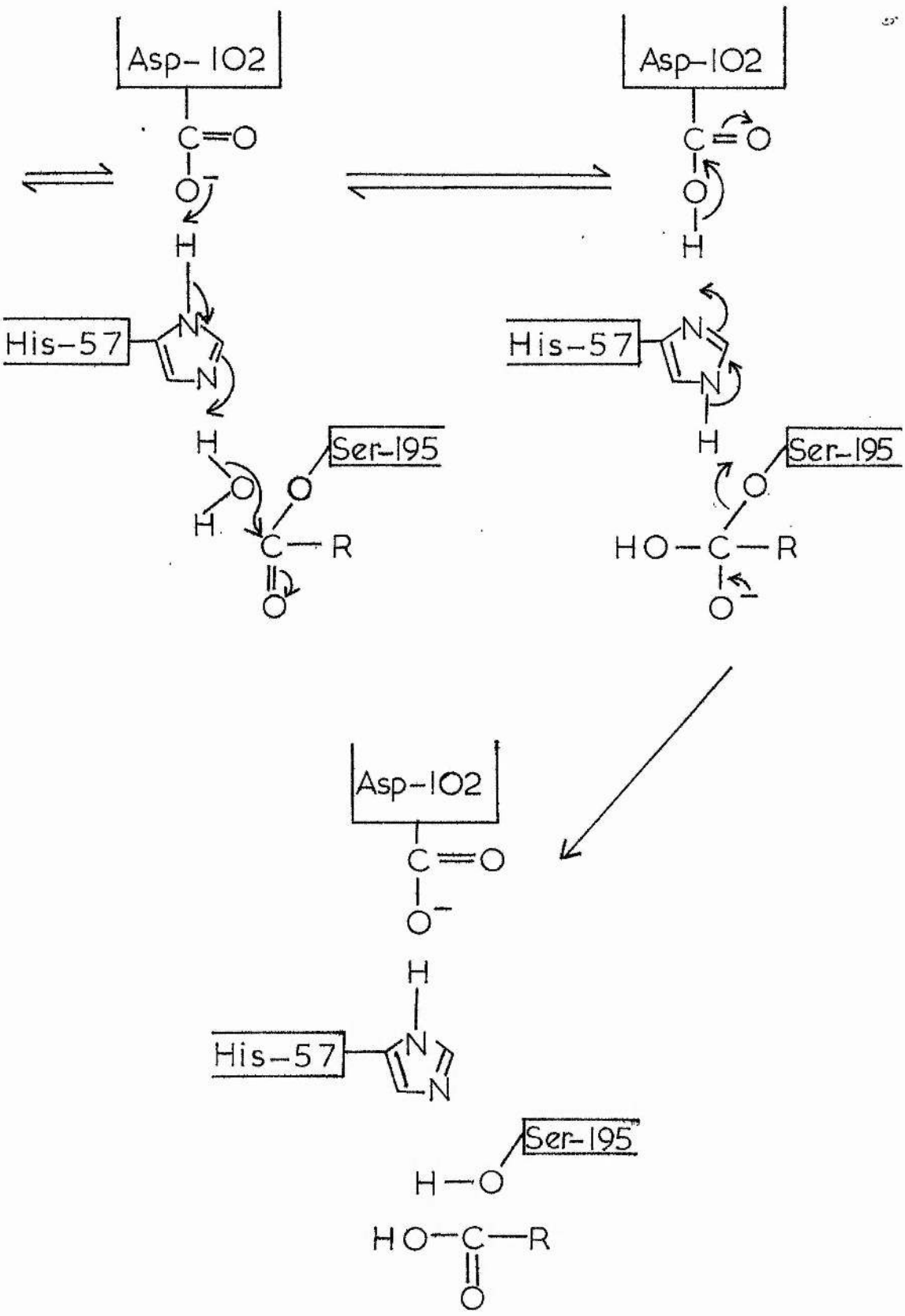
(e.g. Laidler[99]).

The mechanism which is at present best in accord with the evidence observed, is however, that of Blow and co-workers, who have shown[53,97] that there is a buried carboxyl group in α-ct (that of aspartic acid -102) which is hydrogen bonded to the imidazole group of histidine-57. They propose that on ionisation this carboxyl group hydrogen bonds to the imidazole group of histidine-57, which in turn hydrogen bonds to the hydroxyl group of serine-195, making the latter a more powerful nucleophile. This mechanism (Scheme 11), if correct, means that the pK_a of 6.8 is that of the carboxyl group which is hydrogen bonded to the imidazole group.

A consideration of the mechanism of α-ct catalysed hydrolysis should take account not only of the actual bond-breaking process which occurs, but also of the equally important nature of the active site. The mode of binding of the substrate to the enzyme, which is controlled by the environment of the active site, is responsible for the characteristic specificity of the enzyme. The existence of more than one binding site has been postulated[100] for α-ct; following a study of the stereo specific hydrolysis of L-enantiomers, specific and non-specific binding sites have been proposed. Some workers[101] also suggest that for certain mechanisms aromaticity in the substrate is important for the formation of an ion pair between aspartic acid-102 and isoleucine-16 - these residues being in a non-polar region of the active site. The active site has been successfully mapped by Cohen and co-workers[100], and, using this physical structure as a template observed examples of specificity have been explained.


Asp-102
$-H^+$
H^+
His-57
Ser-195
R'O-C-R
O
Asp-102
His-57
Ser-195
R'O-C-R
O
Asp-102
His-57
Ser-195
R'O-C-R
O
Asp-102
His-57
Ser-195
R'OH
R-C
O

Scheme II(i)


Asp-102
His-57
Ser-195
Asp-102
His-57
Ser-195
Asp-102
His-57
Ser-195

Scheme II(ii)

The overall state of knowledge concerning the enzyme α-ct is that, although accurate correlations have been made between the observed catalytic action and the groups known to be involved in this action, there are still doubts as to the precise mechanistic nature of the process. Despite the very considerable amount of previous work concerning α-ct, it was, in conclusion, felt that an attempt to separate steric and electronic substituent effects in a correlation of the reactivities of a series of substituted phenyl acetates might yield interesting kinetic evidence concerning the catalytic mechanism of α-ct.

2. RESULTS AND DISCUSSION

The kinetics of the α-chymotrypsin (α-ct) catalysed hydrolysis of phenyl acetate and a series of substituted phenyl acetates (the substituents being 3-nitro , 4-bromo, 4-chloro, 4-ethoxycarbonyl, 4-nitro, 4-cyano, 2,4-dinitro, 2,6-dinitro, 3,4-dinitro, and 3,5-dinitro) were studied in a phosphate buffer of pH 7.60 by spectrophotometrically monitoring the release of the appropriate phenol. Preliminary observations showed that the kinetics of the reactions generally followed one of two distinguishable patterns. Subsequent studies revealed that the first group - designated Group A and containing substituents 4-nitro, 2,4-dinitro, and 3,4-dinitro - gave kinetics in which there was an initial-burst release of the parent phenol, follwed by a slower, steady liberation of the phenol (Table 24). The initial-burst segment of the kinetics necessitated the use of stopped flow spectrophotometry. whilst the steady state reaction was followed using normal spectroscopic methods. The results were analysed using Gutfreund's method (Appendix 3) which enables the rate constants k_2 and k_3 to be separated (see Scheme 9).

The effect of α-ct concentration upon the steady state rate of hydrolysis of PNPA was studied (Table 25) and Figure 9 shows that there is a linear dependence of this rate on the enzyme concentration. Using approximate initial rate data from these reactions, a plot of enzyme concentration against the concentration of 4-nitrophenol liberated during the initial rapid burst was produced (Table 26, Figure 10). From the gradient of Figure 10, which has a value of 1.09, it is apparent that one mole of α-ct liberates, within

TABLE 24

Kinetic data concerning the α-ct catalysed hydrolysis of various substituted phenyl acetate substrates at 25.3°

(Group A)

I = 0.5 M

pH = 7.60

$[\alpha\text{-ct}]_o = 3.23 \times 10^{-5}$ M

$[\text{substrate}]_o = 1.2 \times 10^{-4}$ M

Substrate	λ_{obs} nm	steady-state rate 10^7. M. s^{-1}	Intercept 10^5 M	k_2 s^{-1}	k_3 $10^2 . s^{-1}$
PNPA	400	5.50	8.5	2.66	1.71
2,4-DNPA	406	9.01	9.7	3.60	3.05
3,4-DNPA	400	7.42	9.3	3.30	2.32

TABLE 25

Effect of Cx-ct concentration on the steady-state hydrolytic reaction of PNPA at 25.3°

I = 0.5 M

pH = 7.60

$[PNPA]_o = 1.2 \times 10^{-4}$ M

λ_{obs} = 400 nm.

ε (phenol) = 1.628×10^4 $l\ mol^{-1}$ at 400 nm

10^5 [Cx-ct] M	steady-state rate. 10^7. M. s^{-1}
0.0	0.0
0.4	0.70
0.8	1.30
1.6	2.55
2.4	3.69
3.2	5.03

TABLE 26

Approximate initial rate data for the reaction between α-ct and PNPA at 25.3°

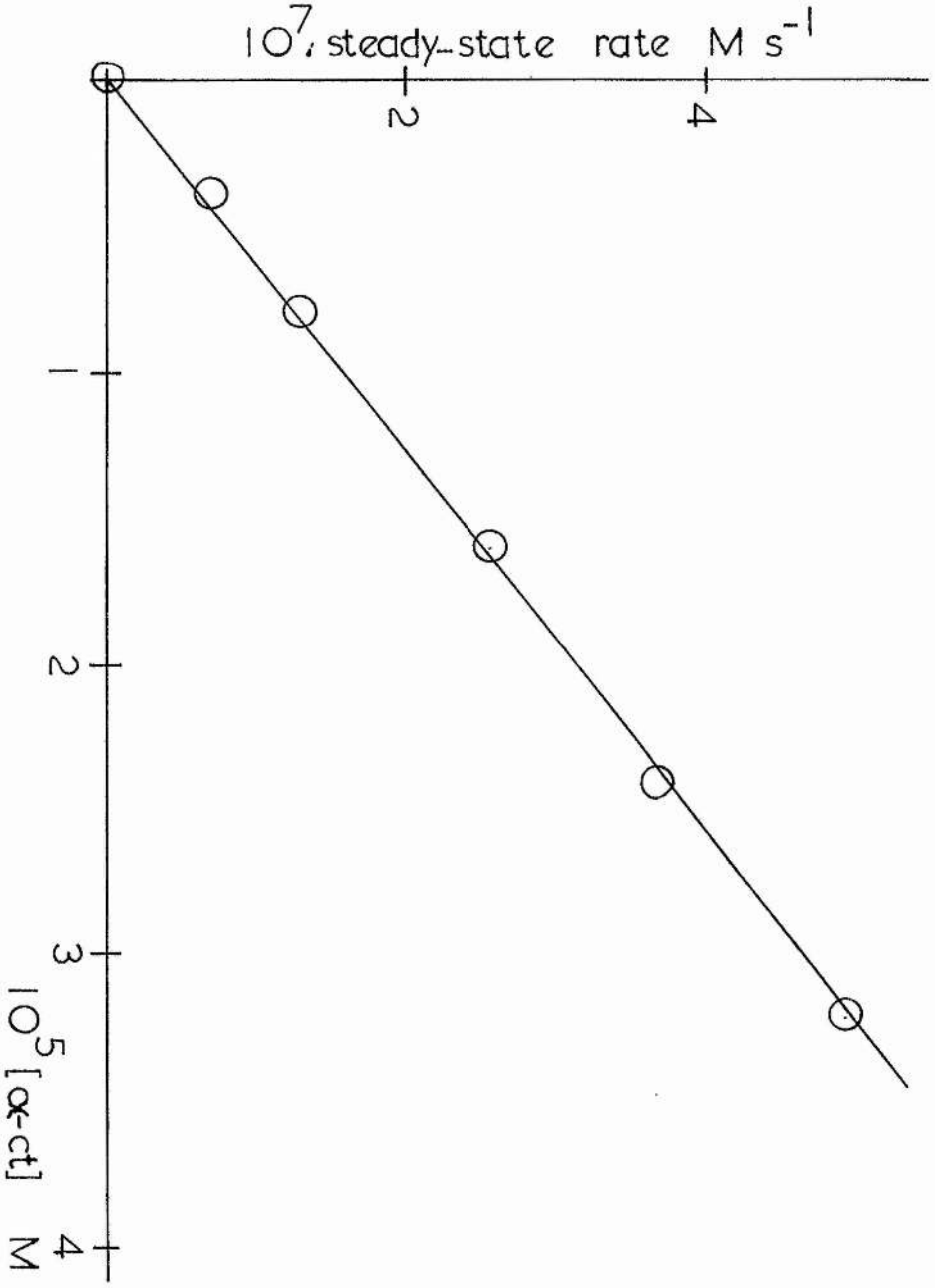
I = 0.5 M

pH = 7.60

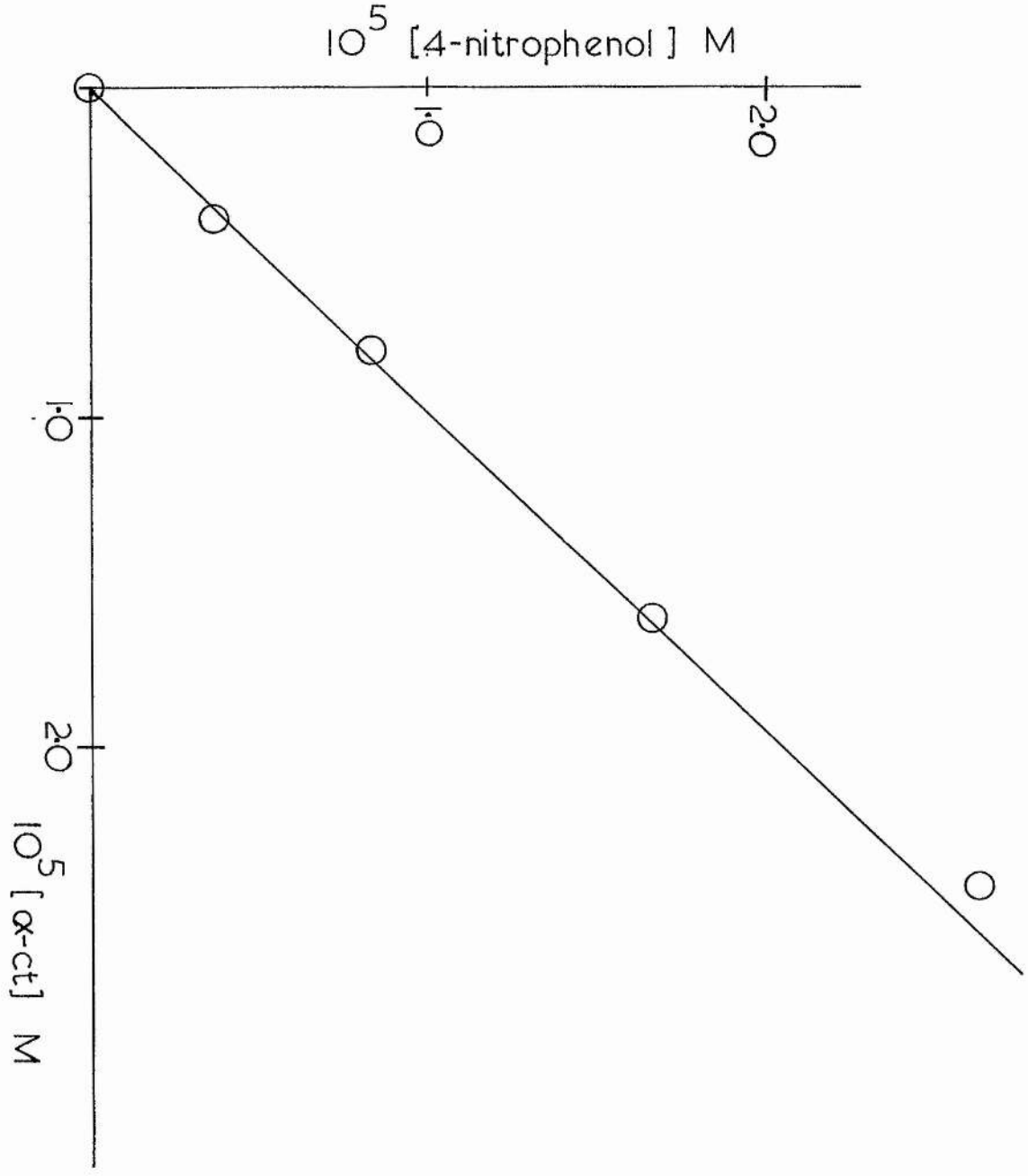
$[PNPA]_o = 1.4 \times 10^{-4}$ M

λ_{obs} = 400 nm

10^5 [α-ct] M	Absorbance,	10^5 [4-nitrophenol] M
0.0	0.0	0.0
0.403	0.06	0.37
0.806	0.14	0.84
1.613	0.27	1.67
2.419	0.43	2.64



(Figure 9 ; Variation of the steady-state rate for the α-ct catalysed hydrolysis of PNPA, at 25.3°, with the concentration of α-ct)

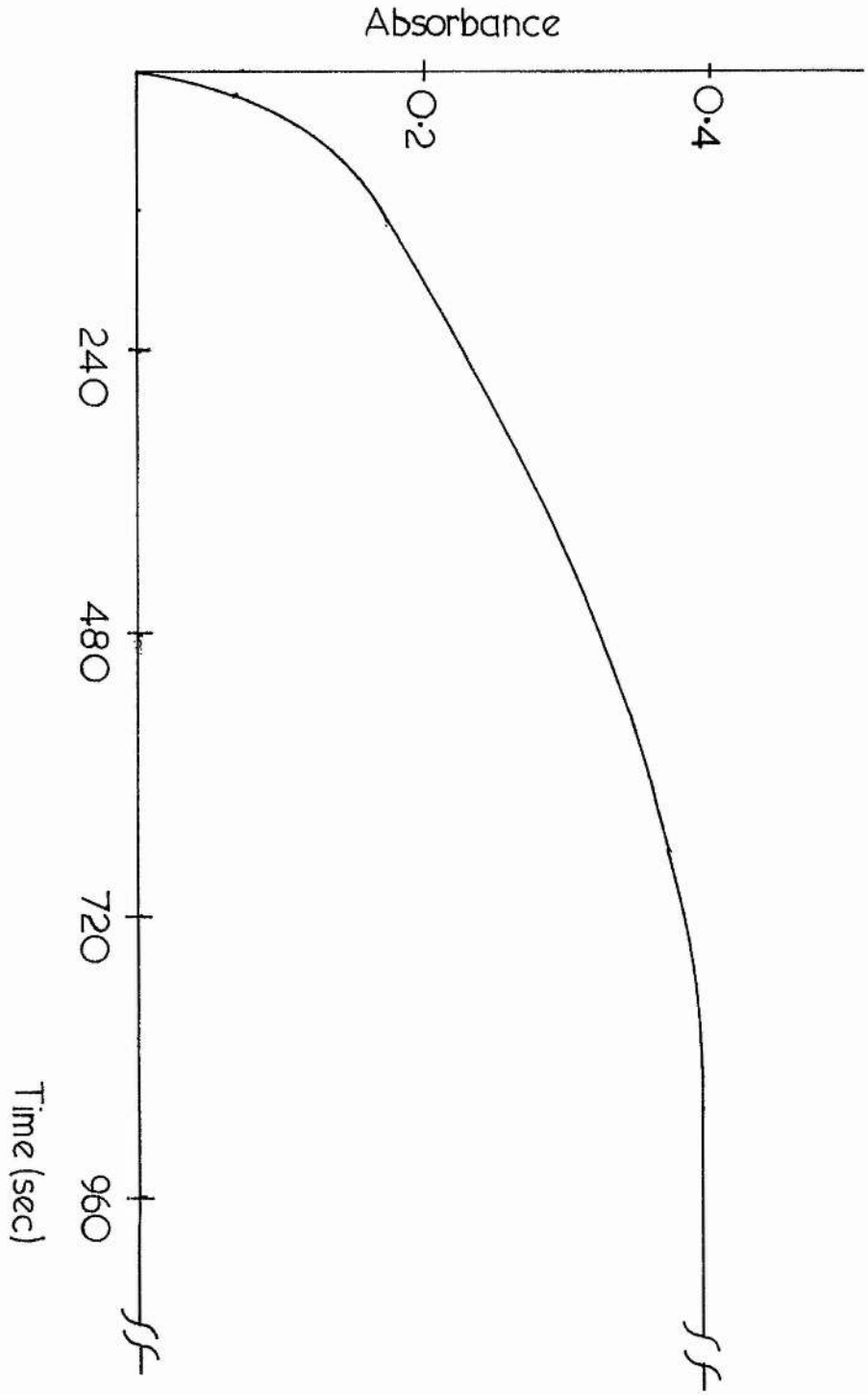


(Figure 10 ; Approximate initial rate data for the α-ct catalysed hydrolysis of PNPA at 25.3°)

experimental error, 1 mole of 4-nitrophenol during the pre-steady state reaction. This result is consistent with k_2 being larger than k_3 ; in such a case, the initial burst corresponds to complete acylation of the enzyme, with a concomitant release of the phenol. Further formation of 4-nitrophenol is controlled by the relatively slow hydrolysis of the acyl enzyme to regenerate the free enzyme. This observation is in accord with previous work by Hartley[100].

The α-ct catalysed reactions of the second group (Group B, comprising the unsubstituted phenyl acetate, and substrates containing 3-nitro, 4-bromo, 4-chloro, 4-ethoxycarbonyl, 2,6-dinitro, and 3,5-dinitro substituents) were studied using conventional spectroscopic techniques. All the reactions gave good first-order kinetics (Table 27) over at least the first three half-lives. Complex kinetics were, however, produced when 4-cyanophenyl acetate was used as a substrate ; the kinetics observed fall into neither of the preceding patterns, being rather a composite of both (Figure11).

The figures in Table 24 indicate that the acylation rate constant (k_2 in Scheme 9) is greater than the deacylation rate constant (k_3). The deacylation rate constant has a similar value for the three substrates used, indicating that, in the catalytic hydrolysis of the three esters, the rate limiting step occurs either before or after the hydrolysis of the esters. It has been observed[102] that the rate limiting step in the α-ct catalysed hydrolysis of three phenylalanine esters was the decomposition of the N-acetyl-L-phenylalanine-enzyme. For the series of aryl acetate substrates used in this work, the acyl-enzyme complex formed is identical in each case ; the rate of decomposition of this common



(Figure 11 ; α-ct catalysed hydrolysis of 4-cyanophenyl acetate at 25.3°)

TABLE 27

Kinetic data concerning the α-ct catalysed hydrolysis of various substituted phenyl acetates at 25.3°

(Group B)

I = 0.5 M

pH = 7.60

$[\alpha\text{-ct}]_o = 3.23 \times 10^{-5}$ M

$[\text{substrate}]_o = 1.2 \times 10^{-4}$ M

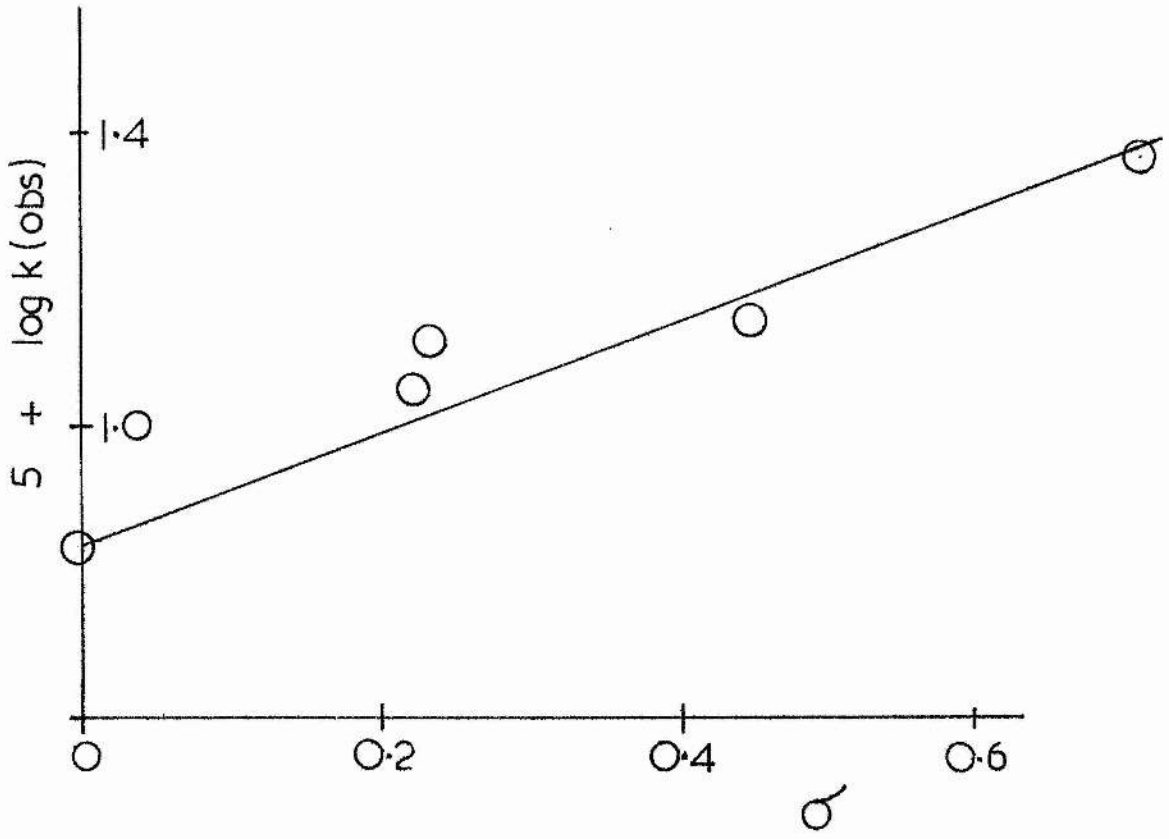
	10^5 k(obs) s^{-1}	σ	λ_{obs} nm
phenyl acetate	6.81	0.0	275
3-nitrophenyl acetate	23.2	+0.710	400
4-ethoxycarbonyl-phenyl acetate	13.8	+ 0.450	294
4-chlorophenyl acetate	11.2	+ 0.227	293
4-bromophenyl acetate	13.1	+ 0.232	278
	k (obs) s^{-1}		
2,6-dinitrophenyl acetate	9.60×10^{-4}		426
3,5-dinitrophenyl acetate	1.97×10^{-2}		400

intermediate would thus be expected to be the same for each substrate.

In Table 27, which shows the first-order rate constants obtained for enzymatic hydrolysis of Group B substrates, the substituent effects upon the reaction rate are such that the data gives a linear Hammett plot (using σ values). The value of ρ obtained from this plot is 0.78 (Figure 12). Studies[46, 103] of imidazole catalysis of esters have shown that effective catalysis by this base is associated with a ρ value of 1.90 : Figure 12, therefore, suggests that imidazole is not directly responsible for the catalytic hydrolysis in this case. Such a conclusion must, however, be treated with circumspection, for it is by no means certain that physical data of this type, obtained in homogeneous aqueous solution, can be directly correlated with the enzymic environment[78].

The sensitivity of the reaction rate to the substituent of the substrate can be understood in terms of the electron-density located at the acyl carbon atom ; 3-nitrophenyl acetate, for instance, has a higher rate of hydrolysis than phenyl acetate because the electron-withdrawing nitro-group in the 3-position facilitates the formation of the phenol. A comparison of Tables 24 and 27, and a consideration of the different types of kinetics observed for each group of substrates, indicates that there is a change in the rate determining step in proceeding from Group A to Group B substrates (i. e. to substrates with improved leaving groups). Group A substrates have the deacylation rate (k_3) as the rate determining step, resulting in the observed kinetic pattern (Figure8), whilst for Group B substrates the acylation rate (k_2) is the limiting factor, and first-order release of the phenol is observed. A correlation of mechanism with pK_a value

(Figure 12 ; Hammett plot for the α-ct catalysed hydrolysis of substituted phenyl acetates at 25.3°)



of the leaving group would predict, however, that 2,6-dinitrophenyl acetate and 3,5-dinitrophenyl acetate would exhibit Group A type kinetics (Table 28) ; the clearly anomalous behaviour will be discussed later.

An analogue computer was used in an attempt to simulate the observed kinetics of both classes of substrates (Appendix 4). By matching analogue traces thus obtained with experimental traces, it appears that Group B kinetics are produced by the postulated Scheme 9 when the ratio k_3/k_2 is greater than one. Group A kinetics are produced when the ratio k_3/k_2 is much less than one. Such evidence supports the change in mechanism postulated above. The kinetics peculiar to 4-cyanophenyl acetate could not be simulated by this method, although some indication was found that the kinetic pattern could be duplicated by changes in the ratio of the rate constants for the reaction with time. The analogue computer - which does not predict absolute values for the rate constants but merely responds to changes in the variable k_3/k_2 - showed that the composition kinetics (Figure 11) would be observed if, as the reaction proceeds, the ratio k_3/k_2 increases until it is larger than one. At this stage, the mechanism would be predicted to reflect the change from a Group A to Group B mechanism. Although the possibility exists that this change is an artifact of the constructed analogue (and it was unfortunately not possible to obtain accurate data for this model, due to difficulties encountered in obtaining a satisfactory time-base to reflect the change in value of k_3/k_2 with time), it is thought that this model does indeed reflect a true picture of the processes occuring during this hydrolysis. One theory of enzymatic action postulates that the enzyme

TABLE 28

Values of pK_a for nitrophenols used in this study

substrate	pK_a	kinetic pattern observed
2,6-DNPA	3.61	Group B
2,4-DNPA	3.97	Group A
3,4-DNPA	5.53	Group A
3,5-DNPA	6.73	Group B
4-NPA	7.14	Group A
3-NPA	8.40	Group B
4-CNPA	7.95	composite

weakens the relevant bond in a substrate by inducing strain within that bond. In some theories this strain is produced by a fast substrate-induced isomerisation of various enzymatic groups. Such mathematical changes in the relative values of the catalytic constants for the 4-cyanophenyl acetate substrate might correspond, in vitro, to the interference, by the substrate, with this conformational change in the enzyme.

The anomalous behaviour of the Group B substrates 2,6-DNPA and 3,5-DNPA cannot, however, be accounted for by a change of rate determining step in Scheme 9 as the leaving group tendencies change. Examination of Table 28 shows (using the pK_a of the liberated phenol as a measure of the leaving tendency of that phenol) that 2,6-DNPA and 3,5-DNPA have pK_a values such that one would predict kinetic patterns in accord with those of Group A substrates. Indeed, the base-catalysed hydrolysis of PNPA, 2,4-DNPA (Tables 9 and 14), 3,4-DNPA, 2,6-DNPA, and 3,5-DNPA (Tables 29 and 30) by both potassium hydroxide and pyridine yield kinetic data such that a plot of log k_c against pK_a is linear. The enzymatic hydrolysis of 2,6-DNPA and 3,5-DNPA therefore appears to be anomalous. The disparity between the predicted and the observed catalytic mechanism can be best understood by consideration of the specificity of α-ct.

Hein and Niemann[104] have shown that the binding sites of α-ct are arranged such that certain structural arrangements are preferentially hydrolysed. An example of one such specific substrate is N-acetyl-L-phenylalanine (Formula 3). Blow and co-workers[105], who have been partially

TABLE 29

Hydrolysis of substituted aryl esters in alkaline solution at 25.3°

I = 0.5 M $[substrate]_o$ = ca. 10^{-5} M

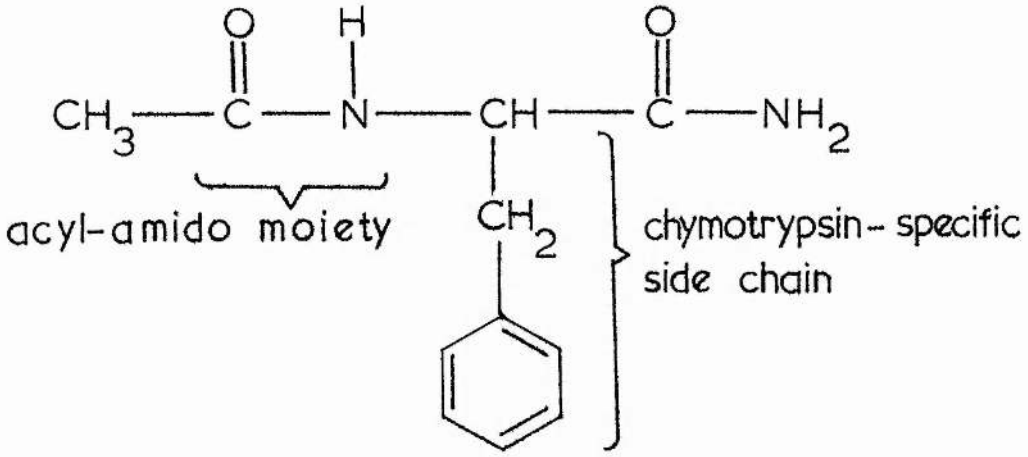
[KOH] M	3,4-DNPA	2,6-DNPA	3,5-DNPA
	k (obs) s^{-1}		
0.1	2.40	6.47	0.93
0.2	4.83	13.12	1.84
0.3	7.31	19.35	2.67
0.4	9.46		3.58
0.5	11.87		4.52
k l mol^{-1} s^{-1}	24.1	64.1	8.9
λ_{obs} nm	430	426	406

TABLE 30

Hydrolysis of substituted aryl esters in pyridine - buffered solution at 25.3°

I = 0.5 M $[substrate]_o$ = ca. 10^{-5} M

$[C_5H_5N]_{free}$ M	3,4-DNPA	2,6-DNPA	3,5-DNPA
	k (obs) s^{-1}		
	x 10^{-2}	x 1	x 10^{-3}
0.080	0.74	0.14	0.65
0.16	1.53	0.30	1.27
0.24	1.82	0.39	1.83
0.32	2.41	0.51	2.25
0.40	3.07	0.63	3.13
k l mol^{-1} s^{-1}	7.59 x 10^{-2}	1.48	7.78 x 10^{-3}
λ_{obs} nm	430	426	285



(Formula 3 ; N-acetyl-L-phenylalanine - an α-ct specific substrate)

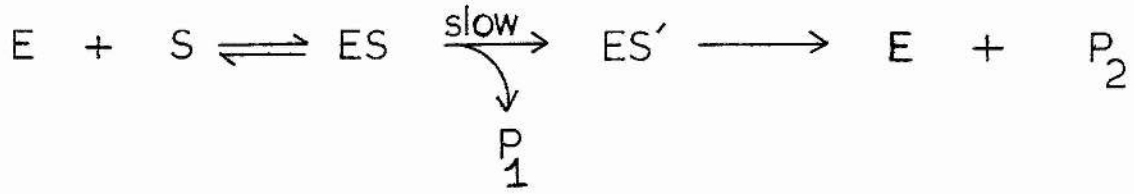
responsible for the physical characterisation of the active site in α-ct, suggest that there is a hydrogen bond between the amido -NH- of the substrate and the carbonyl oxygen of serine-214. Data from X-ray crystallographic studies[106] has shown the existence of cavities around the active site environment which can accommodate an aryl group, an acyl-amide group, a restricted site large enough to hold only the α-hydrogen of a specific substrate, and a hydrolytic site : in such a manner these specific substrates are selectively held and hydrolysed.

It has been proposed[107] that the hydrolysis of specific substrates follow the same mechanism as PNPA and other non-specific substrates. From the data in Tables 24 and 27, however, it is apparent that different substrates have different rate determining steps. The common factor amongst the pseudo-specific substrates in Group A, which separates them structurally from members of Group B, is that they possess a nitro-substituent in the

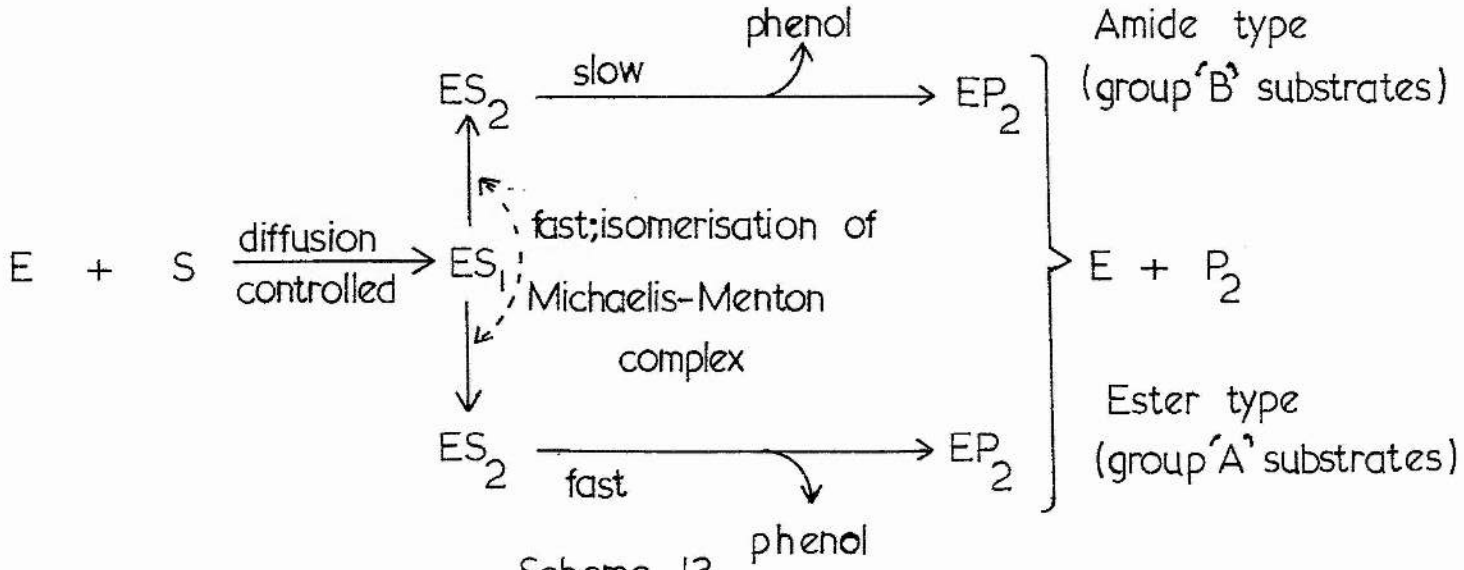
4-position. It is proposed, therefore, that this unique 4-nitrophenyl moiety allows Group A substrates to bind into an enzymic cavity (probably that which accommodates the aryl group of a specific substrate), thus achieving a preferred conformation in which the acylation of the enzyme is a sterically favoured reaction : hence k_2 is large in comparison with k_3. Although 3,5-DNPA and 2,6-DNPA would be predicted to evince kinetics of the same type as Group A substrates, the absence of a 4-nitro-group possibly hinders the achievement of the sterically favoured Michaelis-Menton complex : the incomplete attainment of this state constrains the now comparatively unfavourable acylation reaction to become the rate determining step. Under such circumstances Group B type kinetics are observed (Scheme 12). Where difficulty is experienced in forming an activated complex at the specific site, a non-specific substrate might be hydrolysed at one of the other non-specific sites detected by Blow[100].

Another explanation for the observed catalytic differences might be that the formation of the Michaelis-Menton complex is followed by isomerisation of the enzyme-substrate complex[108]; following this isomerisation, the acylation of the enzyme occurs. Such an isomerization might, as a prerequisite, necessitate the formation of a sterically specific complex (the formation of which is again assisted by a substrate possessing a 4-nitrophenyl group) before full catalytic activity, is attained, and the "specific substrate" kinetic pattern is observed (Scheme 13).

To some extent, the observation of "specific substrate" catalysis by some substituted phenyl acetates is rather fortuitous ; these substrates bear



Scheme 12



Scheme 13

little resemblance to the specific substrates themselves, and the presence of the 4-nitrophenyl group appears to be of much consequence. The α-ct-catalysed hydrolysis of amides demonstrates analogous behaviour to that of Group B substrates in that the acylation rate (k_2) is much smaller than the deacylation rate (k_3)[71]. Gutfreund[109] has observed that the α-ct-catalysed hydrolysis of amides also follows the mechanism shown in Scheme 9 but that, in contrast to "specific substrate" type ester hydrolysis, the rate of formation of the acyl-enzyme is rate-limiting.

It should, however, be noted that the factors which an enzyme can use to influence its catalytic activity are many and varied : orientation effects, removal of water from reactants, bond strain, and acid-base catalysis have all been shown to play a role in α-ct catalysis. The effect of freezing out free rotation around chemical bonds which connect two reacting groups has been demonstrated[110] as having an effect in α-ct catalysis. There is, in view of this multiplicity of factors, no reason to believe that any single factor is of proeminent importance - rather that the many aspects combine together to yield the overall catalytic effect. The use of non-specific ester substrates to study the mechanism of catalytic hydrolysis by α-ct most probably results in subtle shifts of emphasis in the importance of these various factors, and studies, such as this, should be viewed against this background.

3. EXPERIMENTAL

(a) Sources, purification, and preparation of materials

All materials used in kinetic studies were of AnalaR grade.

The enzyme α-chymotrypsin (3 times crystallised, ex. bovine pancreas, and salt-free) was supplied by Koch-Light Laboratories, and was used without further purification.

Preparation of substituted aryl acetates

The substituted aryl acetates used in this study were prepared by the method of Bell, already cited[49] in the Experimental section of Chapter 2. Relevant physical data for these compounds is given in Table 31. The phenols

TABLE 31

Physical data concerning substituted aryl acetates

Substituent	m.p.^{o} or b.p.^{o}	Lit m.p.^{o} or b.p.^{o}	reference	yield %	λ_{obs} nm
unsubstituted	77-79(12 mm)	78-80(10 mm)	114	69	275
4-chloro	140-2(55 mm)	143(55 mm)	80	72	305
4-bromo	150(44 mm)	154(45 mm)	80	67	305
4-cyano	56-57	56	80	59	290
4-ethoxycarbonyl	168(14 mm)			52	294
3,4-dinitro	46	46-47	49	86	430
3,5-dinitro	125-126	125-126	49	93	406
2,6-dinitro	109-110	110-111	49	82	426

used in these preparations were from Ralph Emanuel Ltd. (2,6-dinitrophenol, and 3,4-dinitrophenol), or Koch-Light Ltd. (4-cyano, 4-chloro, 4-bromo, and

phenol), whilst the 3,5-dinitrophenol and the 4-ethoxycarbonylphenol were prepared by the methods given below.

Preparation[112] of 3,5-dinitrophenol

Dry 3,5-dinitroanisole (0.1 mol) was heated to 120° with twice its weight of finely powdered anhydrous aluminium trichloride, and kept at this temperature until the evolution of toxic methyl chloride had ceased (ca. 24 hours). The reaction product was then treated with crushed ice and the resulting solution made strongly alkaline, cooled, and filtered. Acidification, followed by further cooling, precipitated the 3,5-dinitrophenol, which was then twice crystallised from very dilute hydrochloric acid (10^{-3}M) to yield fine colourless crystals. The anhydrous phenol was obtained by heating these crystals, under vacuum, in a drying pistol (60° for two hours). The product had a melting point of 121-122° (lit.,[112] 126°). Yield = 34% .

Microanalysis :-

	%C	%H	%N
Calculated	39.14	2.19	15.22
Found	37.92	2.15	14.93

Preparation of 4-ethoxycarbonylphenol

Ethanol (8 ml), 4-hydroxybenzoic acid (0.05 mol.), and concentrated sulphuric acid (0.1 ml.) were refluxed in benzene (60 ml.) for 8 hours, using a Dean-Stark apparatus. The cooled solution was washed with dilute sodium bicarbonate solution (2 x 25 ml) and water (4 x 25 ml), and then dried over anhydrous calcium sulphate. The solvent was removed under reduced pressure

and the resulting crystalline mass was twice crystallised from chloroform :- melting point, 116^{o} (lit[113] 116^{o}), Yield = 59%.

Microanalytical data for 2,6-DNPA, 3,5-DNPA and 3,4-DNPA

	Calculated %			Found %		
	C	H	N	C	H	N
2,6-DNPA	42.48	2.65	12.39	42.29	2.75	12.46
3,5-DNPA	42.48	2.65	12.39	42.14	2.66	12.51
3,4-DNPA	42.48	2.65	12.39	42.48	2.59	12.54

(b) Kinetic method

The phosphate buffer used in kinetic runs was freshly prepared by mixing potassium dihydrogen phosphate (37.5 ml of 0.5 M solution) and sodium hydroxide (15.7 ml of 1.0 M solution), followed by dilution to 500 ml. with distilled water. This buffer (pH 7.60) had an ionic strength of 0.1 M. The ester solutions used in kinetic studies were freshly prepared before use in spectroscopic grade acetonitrile. Solutions used in stopped flow studies were made up in dilute hydrochloric acid (10^{-4}M) to prevent spontaneous hydrolysis. The ionic strength of all solutions was maintained at 0.5 M by use of potassium chloride.

The kinetic procedures used were identical to those cited in the Experimental section of Chapter 2. In order that the observed changes in optical density of the solutions could be converted into phenol concentrations, the molar extinction coefficients (ε) were measured :

$$\varepsilon = \frac{\text{Optical Density}}{\text{(cell thickness in cm.) x (molar concentration)}}$$

In the case of the stopped flow experiments, the oscilloscope grid was calibrated using buffer solutions of known phenol concentration. All phenols were found to observe Beer's law within the concentration ranges studied.

APPENDIX 1

The derivation of thermodynamic functions from kinetic data

From the Arrhenius equation,

$$k = A \exp^{-E/RT}$$

(where A is the preexponential factor in units of k), a plot of log k versus 1/T yields E (where all temperature values are in units of Kelvin), the Arrhenius energy of activation (cal. mol.$^{-1}$), from the relationship,

$$E = -4.576 \times \text{slope.}$$

Similarly, the enthalpy of activation (units of cal. mol^{-1}) is found from

$$\triangle H^{\ddagger} = E - RT$$

where T is taken as a mean value over the range studied. The entropy of activation, $\triangle S^{\ddagger}$ (units of cal. degree^{-1} or entropy units), may be determined from the relationship,

$$\triangle S^{\ddagger} = 4.576 \log(k/T) + (E/T) - 49.22$$

APPENDIX 2

Method for obtaining the rate coefficient and final concentration of product for a first-order reaction

This mathematical method was developed by Swinbourne[114] and is especially useful when circumstances render conventional determinations tedious or impracticable. If optical density readings $a_1, a_2, \ldots, a_n$ are made at times $t_1, t_2, \ldots, t_n$, and a second series $a'_1, a'_2, \ldots, a'_n$, is made at the corresponding times $t_1 + T, t_2 + T, \ldots, t_n + T$ (where T is a constant) then,

$$(a_\infty - a_n) = (a_\infty - a_o) \exp(-kt_n)$$

and $$(a_\infty - a'_n) = (a_\infty - a_o) \exp[-k(t_n + T)]$$

Dividing one equation by the other, and rearranging $a'_n = a_\infty (1 - \exp. kT) + a_n \exp. (kT)$. By plotting the optical density readings of the first series against those of the second series, a straight line will be obtained ; the rate coefficient may be evaluated from the logarithmic slope of the line. When $t = \infty$, $a_n = a'_n = a_\infty$, and thus the optical density at this boundary limit can be evaluated.

In order to obtain reliable estimates of k and a_∞, the data should be recorded over a period of time greater than two half-lives ($2 \times +t_{\frac{1}{2}}$); T should be in the order of $0.5\ t_{\frac{1}{2}}$ to $t_{\frac{1}{2}}$. It should also be noted that the method is relatively insensitive to deviations from strict first-order law, and it is therefore advisable to undertake a preliminary check to confirm that the reaction under analysis conforms to such a kinetic law.

APPENDIX 3

The evaluation of rate constants involved in enzymatic reactions

This method, developed by Gutfreund[115,116], allows the determination of the rate constants for the acylation process (k_2) and the deacylation process (k_3) from presteady state data for the hydrolytic reaction between α-ct and various specific substrates (see Scheme 9, reproduced below).

(1) $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$

(2) $ES \xrightarrow{k_2} ES' + P_1$

(3) $ES' \xrightarrow{k_3} E + P_2$

It is assumed that c_S (where c_X refers to the concentration of species X) is large enough so that, on the time scale considered, the first transient phase (1) - the formation of the Michaelis - Menton complex - is sufficiently rapid to be considered instantaneous (the singulate perturbation approximation) ; further, that the total enzyme concentration (c_E^o) is equal to c_{ES} plus $c_{ES'}$ (c_E being negligible).

The rate of production of ES' is :-

$$\frac{dc_{ES'}}{dt} = k_2 c_{ES} - k_3 c_{ES'}$$

$$= k_2 c_E^o - (k_2 + k_3) c_{ES'}$$

Rearranging,

$$\frac{d\, c_{ES}'}{\left(\frac{k_2\, c_E^o}{k_2 + k_3} - c_{ES}'\right)} = (k_2 + k_3)\, dt.$$

On integration, $\left[\text{N.B.}, \int \frac{1}{(1 - X)}\, dx = -\log_e (1 - X)\right]$

$$c_{ES}' = \frac{(k_2\, c_E^o)}{(k_2 + k_3)} \left[1 - e^{-(k_2 + k_3)\, t}\right]$$

(Boundary conditions : - $t = o$, $c_{ES}' = o$).

Recall $c_{ES} = c_E^o - c_{ES}'$ and $\frac{dc_{p_1}}{dt} = k_2 c_{ES}$

Hence,

$$c_{ES} = c_E^o - \left(\frac{k_2\, c_E^o}{k_2 + k_3}\right) \left[1 - e^{-(k_2 + k_3)\, t}\right]$$

and so, $$\frac{dc_{p1}}{dt} = k_2 \left\{ c_E^o - \left(\frac{k_2\, c_E^o}{k_2 + k_3}\right) \left[1 - e^{-(k_2 + k_3)\, t}\right] \right\}$$

i. e. $$\frac{dc_{p_1}}{dt} = \frac{k_2\, c_E^o}{k_2 + k_3} \left[k_3 + k_2 e^{-(k_2 + k_3)\, t} \right]$$

Integration gives, (N. B. $\int e^{-x}\, dx = -e^{-x}$)

$$c_{p_1} = \frac{k_3\, k_2\, c_E^o\, t}{k_2 + k_3} - c_E^o \left(\frac{k_2}{k_2 + k_3}\right)^2 e^{-(k_2 + k_3)\, t}$$

The general equation for the production of phenol (p_1) is thus,

$$c_{p_1} = c_E^{o}\left\{ \left(\frac{k_2 k_3}{k_2 + k_3}\right) t + \left(\frac{k_2}{k_2 + k_3}\right)^2 \left[1 - e^{-(k_2 + k_3) t}\right] \right\}$$

For small t, $e^{-(k_2 + k_3) t}$ is approximated by $\left[1 - (k_2 + k_3) t\right]$

Therefore,

$$c_{p_1} = c^{o}_{E}\, k_2\, t \qquad \text{(for small t)}$$

For larger t, $e^{-(k_2 + k_3) t} \longrightarrow 0$ and therefore

$$c_{p_1} \approx c^{o}_{E}\, \frac{k_2 k_3}{k_2 + k_3} \left(t + \frac{k_2}{k_2 + k_3} \right)$$

The expression for c_{p_1} can thus be treated in two ways to correlate with the experimental results.

From the graphs plotted of experimental results, we therefore have the means to separate k_2 and k_3. A specimen trace (that of PNPA and α -ct) is given in Figure 8., and from this data Gutfreund's method yields the values of k_2 and k_3 given in Table 24.

Gutfreund's method of analysis provides a general method for the evaluation of enzymic processes ; many methods have now been developed which deal with more specific cases[117, 118], and sophisticated computational techniques can now be utilised[119].

APPENDIX 4

Calculation of rate constant ratios for enzymatic reactions, utilising analogue techniques

The effect of varying the ratio k_3/k_2 (see Scheme 9) on the type of kinetics observed for the α-ct catalysed hydrolysis of a series of ester substrates was investigated using an analogue computer. The computer (an E.A.I. Pace, model TR-48) does not calculate absolute mathematical values for the rate constants, but merely allows the effect, on the reaction, of varying the ratio k_3/k_2 to be studied. The kinetic analogues thus obtained can be recorded on a chart recorder and compared with the experimentally observed results. In this fashion, it has been possible to show that the differing kinetics observed for Group A and Group B substrates (Chapter 4) are caused by a change in the relative values of k_2 and k_3 (i.e. a change in the ratio k_3/k_2).

The basic computer circuit consists of electronic adders, variable multipliers, and integrators which carry out the mathematical functions. The input of initial conditions is by means of potentiometers, and the rate constants are set by resistance dividers, called constant multipliers. The output is obtained as a trace on an oscilloscope, with a chart recorder link-up. The computer is able to solve the non-linear differential equations which arise in the study of reaction kinetics. The accuracy is comparable to the experimental accuracy for enzyme kinetics (i.e. between one and five percent). A schematic diagram of the analogue computer programme for the Michaelis-Menton mechanism is given in Figure 13.

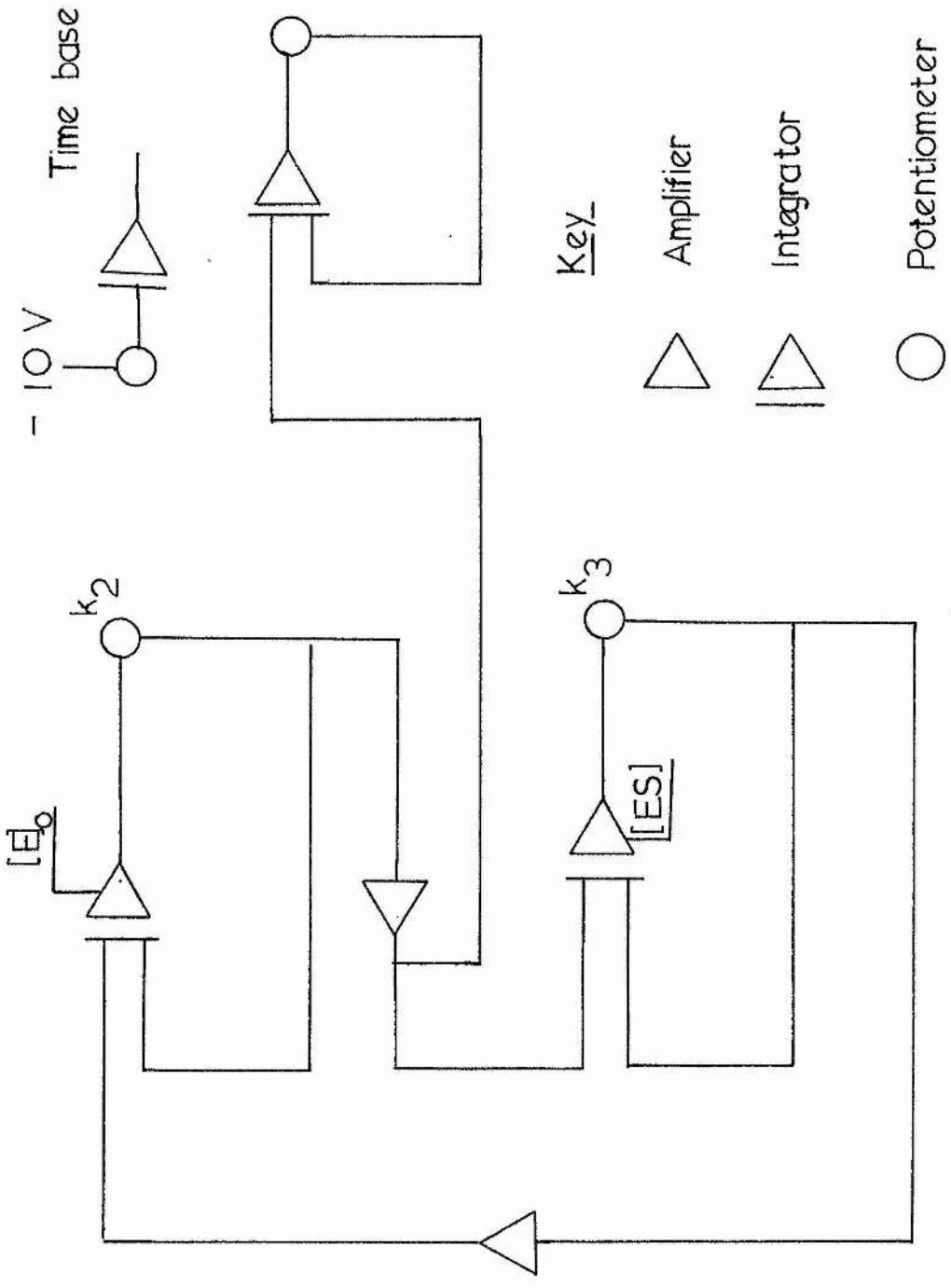

Time base
- 10 V
Key
Amplifier
Integrator
Potentiometer
k_2
k_3
$[E]_o$
[ES]

Figure 13

The simulations made in the programme are of (from Scheme 9) ;

$$\frac{dP_1}{dt} = k_2[E]$$

$$\frac{dE}{dt} = k_3[ES'] - k_2[E]$$

$$\frac{dES'}{dt} = k_2[E] - k_3[ES']$$

The general nature of the curves (see Table 32 and Figure 14, for example) show how a variation in k_3/k_2 ratio effects the kinetics observed. For low k_3/k_2 ratio, k_2 dominates, as would be expected if the slow step is decomposition of the acylated enzyme (as in Group A). At higher ratios, k_2 becomes more important : there is thus evidence for a change in the slow step, from the overall shape of the trace obtained.

From these simulations, a k_3/k_2 ratio of approximately 10/1 or less (i.e. $10/1 < k_3/k_2 < 5/1$) seems to yield Group B type kinetics, whilst smaller ratios give the pattern observed for Group A.

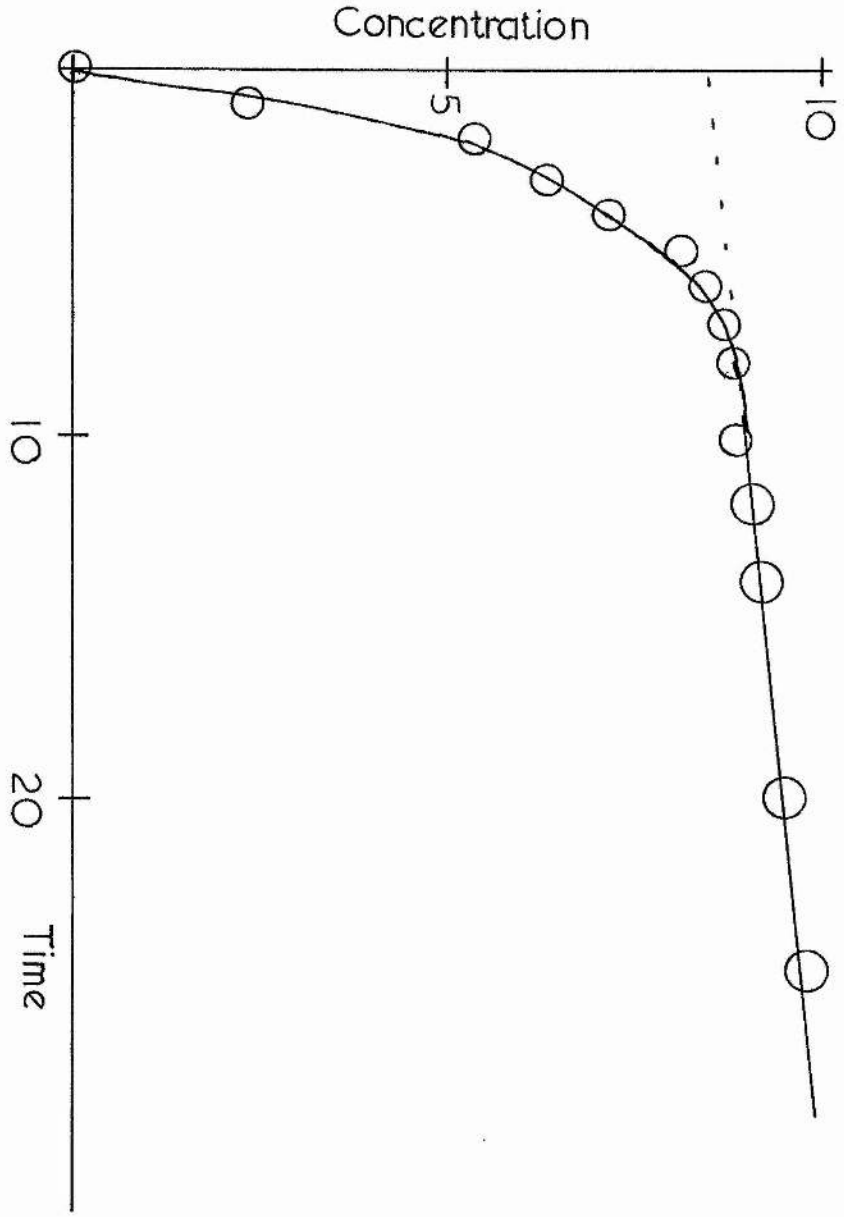
TABLE 32

Analogue data approximating to Group B substrate behaviour

$k_3/k_2 = 10/1$

t time units	0	2	4	6	8	10	12	14	15
c concentration units	0	6.22	10.61	12.44	13.54	14.09	14.45	14.82	15.00

General references for Appendix 4 ; 120, 121, 122



(Figure 14 ; Analogue simulation of observed kinetics of the CX-ct catalysed hydrolysis of Group A substrates)

APPENDIX 5

Catalysis by pyrazole and 4-dimethylaminopyridine in the hydrolysis of 2,4-DNPA

The catalytic effect of pyrazole (Table 34) and 4-dimethylaminopyridine (Table 35) in the hydrolysis of 2,4-DNPA was studied utilising the same methods and experimental details cited in Chapter 2. A comparison (Table 33) of the rate coefficients found, with those found for pyridine bases (Chapter 2), indicates, once again, that a plot of pyridine pK_a against log k is not linear. The catalytic rate constant observed for pyrazole is smaller (9.26×10^{-2} l mol^{-1} s^{-1}) than those observed for the pyridine bases ; such a difference in catalytic effect would be accounted for by the fact that pyrazole is a comparatively weak base (pK_a = ca. 2.5).

TABLE 33

Catalytic coefficients for the base-catalysed hydrolysis of 2,4-DNPA

	$k/l\ mol^{-1}\ s^{-1}$
Pyridine	1.60
3-Methylpyridine	6.50
4-Methylpyridine	8.02
4-Dimethylaminopyridine	124.8
Pyrazole	9.26×10^{-2}

TABLE 34

Hydrolysis of 2,4-DNPA in a pyrazole buffer at 25.3°

I = 0.5 M

$[\text{substrate}]_o$ = ca. 10^{-5} M

λ_{obs} = 350 nm

$[\text{Pyrazole}]_{free}$ M	10^2. k(obs) s^{-1}
0.430	4.08
0.344	3.07
0.258	2.40
0.155	1.45

TABLE 35

Hydrolysis of 2,4-DNPA in a 4-dimethylaminopyridine buffer at 25.3°

I = 0.5 M

$[\text{substrate}]_o$ = ca. 10^{-5} M

λ_{obs} = 406 nm

$[\text{4-dimethylaminopyridine}]_{free}$ M	k(obs) s^{-1}
0.275	34.6
0.222	27.2
0.183	23.4
0.140	17.6
0.105	13.6

APPENDIX 6

Heavy metal ion catalysis of the reaction between 2,4-DNPA and 2(2-hydroxyethyl)-pyridine

The reaction of triethanolamine with nitrophenyl acetates is catalysed by divalent heavy metal ions[123]; rate enhancements of 100 - 1000 have been observed for certain metal ions. The high reactivity of these systems has been shown to be due to the alcoholate group of the metal-bound base, whose pK_a has been lowered by the proximity of the metal ion. The labile nucleophilic alcoholate thus formed attacks the substrate causing its alcoholysis and forming the O-acylated base.

In view of these findings, it seemed reasonable that a base such as 2(2-hydroxyethyl)-pyridine should give a similar rate enhancement in an analogous situation. A series of experiments in which the hydrolysis of 2,4-DNPA was followed (by spectrophotometric monitoring of the appearance of the liberated phenol) in the base-buffer system containing a 0.032 M solution of the metal nitrate under study. The ionic strength was maintained at 0.1 M by addition of sodium nitrate (see Table 36). All runs gave good first-order kinetic plots. Salts of mercury and lead gave precipitation, and hence their kinetics could not be examined.

The results show that copper and iron salts gave rate enhancements the latter by almost a factor of a hundred (over the spontaneous rate). An attempt to determined the nature of the product by the hydroxamic acid method for esters[124] was unsuccessful. It is, however, thought that a similar

TABLE 36

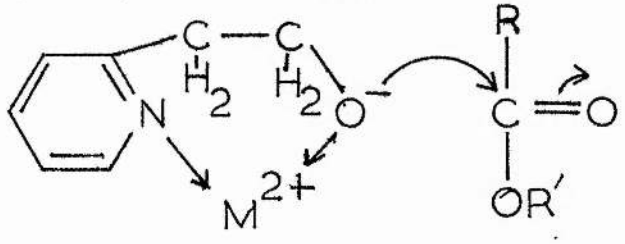
Hydrolysis of 2,4-DNPA in a base-buffer, in the presence of heavy metal salts, at 25.3°

$[base]_{free}$ = 0.064 M I = 0.1 M

$[substrate]_o$ = ca. 10^{-5} M λ_{obs} = 406 nm

Metal	10^4. k(obs). s^{-1}
-	1.04
$Cu(NO_3)_2 . 3H_2O$	4.11
$Co(NO_3)_2 . 6H_2O$	1.11
$Ni(NO_3) . 6H_2O$	0.98
$Sr(NO_3)_2$.	1.43
$Fe(NO_3)_2 . 6H_2O$	93.2
$Ca(NO_3)_2 . 4H_2O$	0.99
$Zn(NO_3)_2 . 6H_2O$	1.01

mechanism operates to that proposed by Shalitin[123] (who did detect an O-acylated product) - see Scheme 14.



(Scheme 14)

In view, however, of previous work with O-acetyl-2(2-hydroxyethyl)-pyridine it is felt that this species would quickly decompose to yield acetate ion and base.

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